IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

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Application of: Lubenow et al.

Serial No.: 09/353,407

Filed: July 15, 1999

Entitled: Methods for Separating Particulate Substrates from Solution While Minimizing Particle Loss

ON APPEAL

ART UNIT: 1655
Examiner: A. Chakrabarti

Atty. Docket No.: QGN-004.0 US

Assistant Commissioner for Patents
Washington, DC 20231

BRIEF ON APPEAL

Sir:

Pursuant to 37 C.F.R. § 1.192, Appellants submit this Brief on Appeal in triplicate, setting forth the basis of their appeal from the Office Action (Paper No. 10), mailed September 18, 2000, finally rejecting Claims 1-66 of the above-identified patent application.

Notice of Appeal pursuant to 37 C.F.R. § 1.191 was filed on December 20, 2000 concurrently with submission of an Amendment in response to the final Office Action.

This Brief is accompanied by the filing fee under 37 C.F.R. § 1.17(c) and the fee to cover an extension from April 20, 2001 (previously paid extension) to the end of a five month extension period under 37 C.F.R. § 1.7(a)(5), making the deadline for filing this Brief July 20, 2001. The Commissioner is authorized to charge any additional fees required in connection with the papers filed herewith to PTO Deposit Account No. 50-0268.

REAL PARTY IN INTEREST

Appellants hereby identify QIAGEN GmbH, a corporation organized and existing under the laws of the Federal Republic of Germany and having an office and place of business in Hilden, Germany, as the assignee of the invention disclosed in the present application.

RELATED APPEALS AND INTERFERENCES

There are no other appeals or interferences related to this appeal known to Appellants.

STATUS OF CLAIMS

The present application was filed with original Claims 1-66.

In the first Office Action on the merits (Paper No. 6), dated March 2, 2000, the Examiner rejected Claims 1-66.

In their response, submitted August 28, 2000, Appellants amended Claims 1, 2, 18, 31, 32, 33, 34, 49, 50, 62, 63, 64, and 66.

In the second Office Action (Paper No. 10), dated September 18, 2000, Examiner finally rejected Claims 1-66.

In an Amendment Under 37 C.F.R. § 1.116 (Paper No. 11), submitted December 18, 2000, Appellants requested cancellation of Claims 1, 4, 9-12, 31, 33, 35, 40-43, without prejudice, and amendments to Claims 2, 3, 14, 15, 16, 23-32, 34, 44-47, 54-64, and 66 to advance the case by claiming particularly preferred embodiments of the invention, i.e., methods of the invention for isolating peptides, polypeptides, or proteins. Appellants' Amendment Under 37 C.F.R. § 1.116 was filed simultaneously with a Notice of Appeal.

In an Advisory Action, dated January 4, 2001 (Paper No. 14), the Examiner informed Appellants of his refusal to enter Appellants' proposed amendments after final and mentioned that even if the amendments were entered that the amended claims would have been obvious over previously cited references. The Advisory Action also informed Appellants that their Notice of Appeal was given a filing date of December 20, 2000.

A set of appealed Claims 1-66 appears in the attached Appendix (Tab A). Claims 1-66 on appeal incorporate the amendments made in Appellants' paper submitted August 28, 2000 but not the amendments submitted after final rejection on December 18, 2000, as those amendments were not entered.

STATUS OF AMENDMENTS

The amendments of Appellants' Amendment, submitted August 28, 2000, have been entered. The amendments of Appellants Amendment Under 37 C.F.R. § 1.116, submitted December 20, 2000 have not been entered.

SUMMARY OF INVENTION

The invention is based on Appellants' discovery of a technique for reducing affinity particle loss in affinity procedures in which the affinity particles are manipulated (e.g., during separation or collection of the particles from a solution). According to the invention, adding detergent to one or more steps in an affinity procedure prior to or contemporaneously with a step in which the affinity particles are manipulated (e.g., collected, separated, or washed) will minimize loss of the affinity particles compared to the same procedure carried out in the absence of detergent (see, e.g., Tables 1-4 and 6 in the Examples section at pp. 20-29 of Appellants' specification). Affinity particles that are treated with detergent according to the invention are believed to form more compact aggregates or pellets when collected or concentrated, and the individual particles tend to adhere less to other surfaces such as walls of tubes and pipettes. Enhanced packing of the detergent-contacted affinity particles decreases particle loss during handling, which, in turn, improves yields of molecules specifically bound by the affinity particles and, thereby, also the reliability and consistency of measurements and assays that employ the affinity particles. Hence, Appellants' claimed invention is an improvement on prior art affinity procedures wherein the affinity particles are manipulated in the absence of detergent, which procedures, as Appellants have demonstrated, are subject to particle loss and, correspondingly reduced and inconsistent yields. A benefit of contacting affinity particles with detergent for the purpose of reducing particle loss during manipulations in an affinity procedure has not been previously described in this art.

SUMMARY OF THE REFERENCES CITED BY THE EXAMINER

The following five references are relied on, alone or in combination, as the basis for the rejections maintained by the Examiner:

1. United States Patent No. 5,942,391, issued Aug. 24, 1999 ("Zhang")
2. United States Patent No. 5,466,577, issued Nov. 14, 1995 ("Weisburg")
3. United States Patent No. 5,646,016, issued Jul. 8, 1997 ("McCoy")
4. United States Patent No. 5,798,442, issued Aug. 25, 1998 ("Gallant")
5. United States Patent No. 4,009,213, issued Feb. 22, 1977 ("Stein")

Zhang

The Zhang patent describes methods for detecting a target nucleic acid from a pathogenic microorganism or from patients with genetic diseases or cancer (see, e.g., col. 3, lines 9-16; col. 5, line 61-col. 6, line 20 of Zhang). The methods of Zhang use multiple nucleic acid probes, including a "capture probe", which is attached to the surface of paramagnetic particles and which binds to a target nucleic acid molecule (see, e.g., col. 3, lines 52-59; Figure 1 of Zhang).

Some examples in Zhang for capturing and amplifying target nucleic acid molecules using streptavidin-coated paramagnetic beads and a buffer, such as lysis buffer, hybridization wash buffer, or a hybridization reaction buffer, which contains a nonionic detergent (see, e.g., col. 27, lines 32-35; col. 28, lines 50-54 and 57-60 of Zhang).

Zhang makes no observation relating to affinity particle loss and makes no mention of how to reduce affinity particle loss in procedures such as the hybridization assays described in that patent.

Weisburg

The Weisburg patent describes nucleic acid probes that hybridize to specific target sequences in the 16S ribosomal RNA of *Borrelia* bacteria, such as *B. burgdorferi*, the etiological agent of Lyme Disease. Weisburg also describes the use of such probes to

detect *Borrelia* target nucleic acid in dot blots (see, e.g., Example 1, col. 6, line 66-col. 7, line 31 of Weisburg) and in sandwich hybridization schemes where a "capture" probe binds a target sequence and a "detector" probe signals the binding of the target sequence (see, e.g., Example 2, col. 7, lines 35-62). Example 3 of Weisburg describes a sandwich hybridization protocol to diagnose Lyme disease using a capture probe linked to a magnetic particle (see, col. 8, lines 10-23). The Examiner appears to rely on Weisburg because of the teaching that sodium dodecyl sulfate (SDS) detergent may be used in a lysis buffer to release RNA from blood cells in a blood sample as a prelude to the hybridization protocol described in Example 3 (see, col. 8, lines 1-6 of Weisburg).

However, Weisburg does not recognize the problem of, or provide a solution for, reducing affinity particle loss in affinity procedures in which the particles are manipulated in the absence of a detergent. Nowhere does Weisburg teach or suggest the improvement of using a detergent in an affinity separation that utilizes affinity particles, in order to prevent or reduce loss of affinity particles during manipulations of the affinity particles in the various steps of the procedure.

McCoy

The secondary reference, McCoy, describes methods for modifying a protein to make it bind more readily to a metal affinity matrix, i.e., by including a "patch" of histidine amino acid residues in a fusion partner for the protein (see, e.g., col. 3, lines 24-31; col. 3, line 46-col. 4, line 6; col. 10, lines 56-66 of McCoy). This reference is apparently cited by the Examiner to demonstrate that a fusion protein having a metal affinity binding patch of histidines may be substituted in whatever affinity separation method is taught by Zhang (see, Paper No. 10, paragraph bridging pages 7-8).

McCoy does not recognize the problem of, or provide a solution for, reducing affinity particle loss in affinity procedures in which the particles are manipulated in the absence of a detergent. Nowhere does McCoy teach or suggest the improvement of using a detergent in an affinity separation that utilizes affinity particles, in order to prevent or reduce loss of affinity particles during manipulations of the affinity particles in the various steps of the procedure.

Gallant

The Gallant patent describes isolation of "apopain", a protease which appears to play a key role in the onset of apoptosis, and tripeptidyl derivative compounds that inhibit apopain (see, e.g., col. 1, lines 6-15; col. 10, lines 17-col. 14, line 56 of Gallant). The Examiner appears to rely on Gallant (Paper No. 10, paragraph bridging pages 8-9) for its mention of the use of the zwitterionic detergent CHAPS in an HPLC purification and a streptavidin-agarose purification (see, col. 22, lines 33-46, and col. 23, lines 3-19 of Gallant).

The section of Gallant specifically cited by the Examiner describes a purification scheme for apopain using various column chromatography steps. For example, the purification scheme described in column 22 of Gallant involves applying a cytosolic fraction of THP-1 cells to a DEAE-5PW ion exchange HPLC column that had been pre-equilibrated in a Tris/HCl buffer comprising 0.1% (w/v) CHAPS zwitterionic detergent. Proteins were then eluted from the column with a linear gradient of NaCl in Tris/HCl buffer also comprising 0.1% (w/v) CHAPS. However, it is clear that the affinity particles in the HPLC column are never manipulated because they remain immobilized in the column.

Thus, the secondary Gallant reference does not recognize the problem of, or provide a solution for, reducing affinity particle loss in affinity procedures in which the particles are manipulated in the absence of a detergent. Nowhere does Gallant teach or suggest the improvement of using a detergent in an affinity separation that utilizes affinity particles, in order to prevent or reduce loss of affinity particles during manipulations of the affinity particles in the various steps of the procedure.

Stein

The secondary Stein reference is directed to an improvement of a continuous process for separating lipid mixtures, such as fatty alcohols, of different melting points based on a solid and oil phase separation of each component lipid, which process includes converting the fatty alcohol components in a mixture into different forms that have different melting points, dispersing solid forms and liquid forms of fatty alcohols using a "wetting agent", and then drawing off a liquid oil phase containing a component

fatty alcohol from the dispersed solid forms of other converted fatty alcohol compounds (see, e.g., col. 3, line 55-col. 4, line 2 of Stein). According to Stein, a variety of "wetting agents" may be used to disperse solid and oil phases that are subsequently separated in the continuous process (see, e.g., col. 5, line 59-col. 6, line 24 of Stein). The Examiner apparently relies on Stein for its mention of the cationic detergent dodecyl trimethyl ammonium chloride, which is an example of a wetting agent that may be incorporated in the continuous process of separating mixtures of fatty alcohols (see, e.g., col. 6, lines 22-24 of Stein). However, the improved method of continuous separation of lipid mixtures described by Stein does not involve use of affinity particles.

Thus, the Stein reference does not recognize the problem of, or provide a solution for, reducing affinity particle loss in affinity procedures in which the particles are manipulated in the absence of a detergent. Nowhere does Stein teach or suggest the improvement of using a detergent in an affinity separation that utilizes affinity particles, in order to prevent or reduce loss of affinity particles during manipulations of the affinity particles in the various steps of the procedure.

ISSUES ON APPEAL

The issues remaining after the final rejection (Paper No. 10) and the Advisory Action (Paper No. 14) are:

I. Whether Zhang describes the invention defined in appealed Claims 1-4, 9, 13-19, 23, 24, 31-35, 44-50, 54, 55, and 62-66 in the manner prohibited under 35 U.S.C. § 102(e)?

II. Whether Weisburg anticipates the invention defined in appealed Claims 1-4, 9, 13-17, 20, 33-35, 44-46, 48, 49, and 64-66 in the manner prohibited under 35 U.S.C. § 102(b)?

III. Whether the invention as defined in appealed Claims 1-4, 9, 13-17, 20, 25, 26, 33-35, 44-49, 56, 57, and 64-66 would, at the time of the invention, have been obvious under 35 U.S.C. § 103(a) to a person of ordinary skill in the art in view of the teachings of Weisburg?

IV. Whether the invention as defined in appealed Claims 1-19, 23, 24, 31-50, 54, 55, and 62-66 would, at the time of the invention, have been obvious under 35 U.S.C. §

103(a) to a person of ordinary skill in the art in view of the combined teachings of Zhang in view of McCoy?

V. Whether the invention as defined in Claims 1-4, 9, 13-19, 21, 23, 29-35, 44-50, 52, 54, 55, and 60-66 would, at the time of the invention, have been obvious under 35 U.S.C. § 103(a) to a person of ordinary skill in the art in view of the combined teachings of Zhang in view of Gallant?

VI. Whether the invention as defined in appealed Claims 1-4, 9, 13-19, 22, 27-29, 31-35, 44-50, 52, 53-55, and 58-66 would, at the time of the invention, have been obvious under 35 U.S.C. § 103(a) to a person of ordinary skill in the art in view of the combined teachings of Zhang in view of Stein?

GROUPING OF THE CLAIMS

In the appealed claims, Claims 1, 2, 33, 34, 64 and 66 are independent claims. Claims 2, 34, 64 and 66 are in Jepson format, which emphasizes that the invention is an improvement of known affinity purification methods.

None of the grounds of final rejection distinguishes between the Jepson format claims and the other independent claims, as all of the independent claims are subject to all of the rejections. Accordingly, with respect to the grounds of rejection as set forth in the Office Action of September 18, 2000 (Paper No. 10) and in the Advisory Action of January 4, 2001 (Paper No. 14), Appellants believe that Claims 1-66 stand or fall together.

However, if the Board determines that the Jepson format claims (Claims 2, 34, 64 and 66) are to be accorded a different scope than the other independent claims (i.e., Claims 1 and 33), interpreting such claims in light of the specification and prosecution history thus far, then Appellants assert that the Jepson format claims are separately patentable for that reason, and request that for each ground of rejection the Jepson format claims be considered separately from the other independent claims.

ARGUMENTS

Introduction

All of the claims of the present application are directed to an improvement in affinity separation methods utilizing affinity particles wherein the loss of affinity particles in the course of running the affinity separation is minimized via the exposure of the affinity particles to a detergent. Independent Claims 2, 34, 64 and 66 have been written in Jepson form in order to emphasize the nature of the improvement and the context in which Appellants' discovery is inventive.

The Examiner has not cited any prior art that pertains to affinity separations in particular. All of the citations relate to various protein or nucleic acid products or their use in assays, and in the midst of the text of these citations non-specific reference is made to detergent being present in a buffer used in a purification process or assay procedure.

No buffer compositions are claimed by Appellants. All of the appealed claims are directed to methods, i.e., methods of isolating a target molecule using affinity particles (e.g., magnetic affinity beads).

As Appellants' will discuss in detail below, none of the references teaches the art anything about the technical problem that inspired Appellants' invention, and none of the references, alone or in any combination, teaches a person skilled in the art how to modify an affinity separation method to reduce the loss of affinity particles. With respect to the particular invention *as it is claimed* by Appellants, it is respectfully submitted that all of the citations applied against the claims are irrelevant, and as a consequence the final rejection of the claims should be REVERSED by the Board.

1. The problem solved by Appellants' claimed invention is not disclosed, suggested, contemplated or understood in any reference or combination of references relied on by the Examiner

Appellants' claimed invention is an improvement over prior art methods to separate or isolate molecules of interest in solutions using affinity particles that bind to the molecules of interest. As explained in Appellants' application at page 3, lines 15-24, such prior art affinity separation methods are typically plagued by the loss of some affinity particles during the manipulations of the particles that must be performed (e.g.,

separation of the particles from a solution, washing, and transferring the particles from one solution to another). The loss of particles during the process steps leads to reduced yields and inconsistent purification or assay results. However, according to Appellants' invention, contacting the affinity particles with detergent prior to such manipulations reduces particle loss and thereby provides greater yields and reproducibility of results (see, Examples 1-6 and Tables 1-6 at pp. 20-29 of Appellants' specification). Thus, Appellants' claimed invention improves all prior art affinity procedures wherein the affinity particles are manipulated in the absence of detergent. As explained below, none of the references relied on by the Examiner, either alone or in combination, provides any substantial evidence of knowledge of the problem of how to prevent or reduce loss of affinity particles during manipulations of the particles in the absence of detergent.

The Zhang reference describes the use of multiple nucleic acid probes to detect target nucleic acids in a sample, wherein one of the probes, i.e., a "capture-amplification probe", can be linked to paramagnetic particles to separate the target nucleic acids from the sample. Zhang provides no indication of an appreciation for or a knowledge of the difference in using affinity particles that have or have not been exposed to a detergent.

The Examiner referred in particular to Examples 1 and 9 of Zhang as evidence of a teaching or a suggestion of Appellants' claimed methods. In Example 1 of Zhang, non-ionic detergent is used in a lysis buffer containing the nucleic acid probes and later in a particular buffer used to wash away any unbound proteins, nucleic acids, and probes that may have been trapped non-specifically in the hybridization complex. The complex is also linked via the capture-amplification probe to affinity particles (see, col. 27, lines 31-52 of Zhang). In Example 9 of Zhang, another detergent is used in a hybridization buffer and wash buffer for the hybridization complex (see, col. 39, line-col. 40, line 8 of Zhang).

Appellants are well aware that various types of cell lysis buffers, hybridization buffers, and wash buffers with or without detergent have been developed and used in molecular and cellular biology for decades (see, e.g., Grunstein and Hogness, *Proc. Natl. Acad. Sci. USA, 72*: 3961-3965 (1975) (Exhibit A); Southern, *J. Mol. Biol., 98*: 503-517 (1975) (Exhibit B); pp. 1.101-1.103, 9.45, and 9.52-9.54 (containing excerpts from various nucleic acid hybridization protocols) *In* Sambrook et al., Molecular Cloning: A Laboratory Manual (second edition), (Cold Spring Harbor Laboratory Press, Cold Spring

Harbor, 1989) in Exhibit C; pp. 577-578 (excerpts describing various methods of lysing cells) *In* Zubay, Biochemistry (Addison-Wesley Publishing Co., Reading, MA 1983) in Exhibit D; pp. 18,30-18.40 (excerpts describing various methods of lysing cells) *In* Sambrook et al., Molecular Cloning: A Laboratory Manual (second edition), (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 1989) in Exhibit E)). However, Zhang's description of employing such standard buffers and nucleic acid hybridization procedures provides no recognition of the problem of particle loss during manipulations of the particles in the absence of detergents or any insight to resolving this problem. Accordingly, Zhang only provides a reiteration of prior art buffers and procedures used in molecular biology. The advantage of Appellants' claimed invention is unrecognized in the Zhang reference, and therefore Zhang cannot be regarded as providing a teaching relevant to the invention *as claimed.*

Weisburg is also void of any appreciation of the problem of losing affinity particles in prior art procedures that require handling of affinity particles or any appreciation of a means for its resolution. The Weisburg patent describes nucleic acid probes for the detection of 16S ribosomal RNA (rRNA) sequences which are specific for the Lyme disease causing bacteria, i.e., *Borrelia burgdorferi*, and, under certain hybridization conditions, related species as well (see, e.g.,col. 5, line 49-col. 6, line 58 of Weisburg). Weisburg provides a description of using the probes in a sandwich assay in which a "capture probe" containing a nucleic acid sequence specific for a target 16S rRNA of a bacterial species of *Borrelia* is tethered by standard A-T base pair homopolyermic sequences to a solid surface, such as a glass bead, filter disc, or magnetic particle (see, e.g., Fig. 1; col. 7, lines 36-50).

The Examiner relied on Weisburg based on a section of the text that states:

> "Blood is preferably processed so as to yield total nucleic acid content, such as by sonication, vortexing with glass beads, *detergent lysis using an agent such as SDS* or chemical treatment, or alternatively, bacterial cells are partially purified by, for example, the DuPont Isolator System, followed by cell lysis. Probe 1620, with a polymeric stretch of Adenosine residues attached, and Probe 1621 including a 32-Phosphorous tag, are then ideally combined with the sample in chaotropic buffer such as guanidinium isothiocyanate. Magnetic particle beads

> which have been derivatized with oligo-Thymidine also are advantageously included." (col. 8, lines 1-12 of Weisburg, emphasis added)

The use of detergent to lyse blood cells is one of many known methods to lyse cells available in the art. Other techniques include osmotic shock, alkali treatment (e.g., with NaOH), enzymatic digestions, and sonication, depending on the type of cells and the needs of the practitioner (see, e.g., Exhibits D and E). Thus, the use of detergent in buffers to lyse cells as described in Weisburg provides no teaching relating to the problem of affinity particle loss during affinity separation procedures, nor does Weisburg teach or suggest the benefit of contacting affinity particles with detergent prior to the step of manipulating the particles to reduce particle loss compared to prior art affinity procedures performed in the absence of detergent.

McCoy, Gallant, and Stein also fail to recognize the problem of losing particles during manipulations in prior art affinity separation procedures. Appellants also note that there is no substantial evidence of a motivation or teaching in any of these references or in Zhang for these references to be combined to serve as a basis for rejecting the claims on appeal. The patent law forbids such unmotivated combinations of references as hindsight reconstruction, so that rejections based on such combinations are improper. *See, In re Kotzab*, 217 F.3d 1365, 55 USPQ2d 1313 (Fed. Cir. 2000). However, even if the references are combined, persons of ordinary skill in the art are still not provided with an appreciation of the problem of loss of affinity particles during the steps of prior art affinity separation protocols, nor are they provided with a teaching or a suggestion of Appellants' method for solving the problem.

McCoy describes fusion proteins in which a thioredoxin or thioredoxin-like protein is fused to a protein of interest to provide stability to the protein of interest during synthesis in *E. coli* bacteria. According to McCoy, such proteins may be engineered to enhance their binding to metal binding/chelating columns. However, *McCoy does not even describe an example of manipulating affinity particles*. McCoy only describes protocols for using prior art metal binding/chelating affinity **columns** in the absence of detergent (see, e.g., col. 16, lines 55-67; col. 30, lines 24-50 of McCoy).

Likewise, the Gallant reference *does not refer to a separation procedure in which affinity particles are manipulated* (i.e., suspended, washed, transferred, collected). Gallant describes a routine protocol for pre-equilibrating an anion exchange high performance liquid chromatography (HPLC) **column** using a buffer containing 0.1% (w/v) CHAPS detergent (see, col. 22, lines 33-41 of Gallant). Thus, the anion exchange particles are immobilized in the HPLC column and never subject to handling and loss.

Thus, even if combined with Zhang or Weisburg, the overall teaching of the combination of Zhang (or Weisburg) in view of McCoy, or of Zhang (or Weisburg) in view of Gallant, is similarly deficient, amounting to only a disparate collection of standard lysis, wash, and hybridization buffers using nucleic acid probes from Zhang and either a standard affinity column protocol for proteins engineered to adhere to such columns from McCoy or a standard HPLC ion exchange column protocol for purifying a particular protease from Gallant.

The combination of Zhang and Stein also fails to inform persons of ordinary skill in the art of Appellants' claimed invention. As noted above, Zhang describes use of standard lysis, wash, and hybridization buffers for isolating nucleic acids with several types of nucleic acid probes. Stein cannot cure the deficiencies of Zhang because Stein *makes no mention or suggestion of ANY affinity method*. Stein only describes a continuous method of separating component fatty alcohols from a mixture of lipids that have different melting temperatures. The solid phase fatty compounds are dispersed in the mixture using a "wetting agent" (such as soaps and other surface tension lowering compounds, see, col. 5, line 59-col. 6, line 24 of Stein). Each fatty component is melted into oily liquid form and drawn out of the mixture with a minimal loss of wetting agent in the process (see, e.g., col. 3, line 13-43; col. 3, line 55-col. 4, line 10 of Stein). Stein cannot cure the deficiency of Zhang to suggest Appellants' claimed methods, because Stein is devoid of any a teaching or suggestion of any affinity procedure.

Furthermore, Appellants submit that persons of ordinary skill in the art who are aware of the problem of particle loss during manipulations in affinity procedures would have no reasonable basis to seek an answer to the problem in Stein's improvement on a continuous industrial process for separating fatty compounds based on different melting points. As with the other combinations made by the Examiner, the combination of Zhang

and Stein fails to provide an appreciation for the problem addressed and solved by Appellants' claimed invention.

The Examiner has argued that Appellants have erred by analyzing the references individually, where some of the rejections are based on a combination of references. In the present case, there is no reference of record that either recognizes the technical problem addressed by the invention, no reference that includes a hint of a teaching relating to modification of standard affinity purification protocols, and in fact no reference that relates directly to affinity separation procedures at all. In view of this reliance by the Examiner on art that is not even in the field of the invention, Appellants need not exhaustively analyze the combined teachings of the references. Zero + zero is still zero: that is, since there is no teaching relevant to the modification of affinity separation methods contained in any reference cited by the Examiner, any individual reference OR ANY COMBINATION of such references remains devoid of a relevant teaching.

Clearly, no combination of the references cited by the Examiner is able to provide any recognition of the problem of, or Appellants' solution to, particle loss during manipulations of affinity particles in affinity separation methods. Accordingly, it is respectfully submitted that each of the final rejections applied against Appellants' claims should be reversed.

2. Accidental practice of the claimed invention in the references is insufficient to anticipate or make obvious the rejected claims under 35 U.S.C. § 102 or § 103, respectively.

As noted above, no reference or combination of references relied on by the Examiner either recognizes the problem of losing affinity particles during manipulations in the absence of detergent or the advantage of using Appellants' claimed methods that employ detergent to reduce such affinity particle loss. However, the Examiner also stated that Appellants' claimed methods are an inherent feature of the references:

> "Both Zhang and Weisburg teach exactly the same structure and chemical composition of the claimed invention. Chemicals having same structure and

> composition inherently contains [sic] the capability of performing the intended use recited in the claims." (Office Action of September 18, 2000 (Paper No. 10) at page 10).

The rule to follow regarding the issues of inherency, anticipation, and obviousness, is provided in *In re Zierden*, 411 F.2d 1325, 162 USPQ 102 (C.C.P.A. 1969). In *Zierden*, the appellants claimed a method for removing or preventing alluvium (silt, mud, organic wastes and accumulations) deposits in water systems (such as industrial heat exchange surfaces) comprising adding insoluble potassium metaphosphate and a solubilizing agent therefor. 411 F.2d at 1326-1327, 162 USPQ at 103. The examiner rejected the claims as anticipated under 35 U.S.C. § 102(b) and obvious under 35 U.S.C. § 103 based on French Patent No. 901,765 ("the French patent"), which disclosed the addition of insoluble potassium metaphosphate and a solublizing agent therefor to "industrial waters" (such as heating and cooling systems) to prevent deposition of calcium carbonate scale in such systems. 411 F.2d at 1327, 162 USPQ at 103. The court noted that:

> "Scale and alluvium have different origins and causes and cannot always be prevented or removed in the same ways. This is clearly shown by appellant's specification and by the prior art references.
>
> "The peculiar -- if not unique -- aspect of this case is that it so happens that the scale prevention method and compositions of the French patent are also effective to remove and prevent alluvium accumulations in water systems. The question here is whether that suffices to negative the patentability of the appealed claims. A corollary question . . . *is whether those skilled in the art would know, from the teaching of the French patent, that its scale prevention technique is also effective to prevent or remove deposits of alluvium, the latter being appellant's discovery.* These are the question we must decide." *Zierden*, 411 F.2d at 1327-1328, 162 USPQ 102, 103 (emphasis added).

The solicitor for the Patent Office argued that the French patent in combination with secondary references made clear that industrial waters contained alluvium so that

appellant's discovery was inherent in the French patent. This point was countered by the appellant's view that the case "presents a classic example of accidental use, unrecognized by the art, without profit to the art, and therefore without legal significance as an anticipation," citing *Pittsburgh Iron & Steel Foundries Co. v. Seaman-Sleeth Co.*, 248 F. 705 (3rd Cir. 1917), which the court agreed was on point. 411 F.2d at 1329, 162 USPQ at 105.

The court's analysis began with the recognition that there is an express statutory authority for a patent on a process, which is a new use of a known process (35 U.S.C. § 100). The court then examined the French patent and noted that:

> 1. the French patent contained no account of the phenomenon (alluvium deposits) that appellant's claimed to address, i.e., was not suggested by the prior art reference
>
> 2. there was a need to remove alluvium deposits and current methods differed from appellant's claimed discovery
>
> 3. the record did not show that the French patent provided the obvious solution to the problem at the time of its invention. (*see,* 411 F.2d at 1330, 162 USPQ at 105)

Based on the above points, the court concluded that it could not find the process as claimed obvious or anticipated by the French patent and reversed the rejections of the method claims. *See,* 411 F.2d at 1331, 162 USPQ at 106.

Appellants submit that following the analysis and rule of the *Zierden* case, the claims in this appeal are neither anticipated nor obvious by any combination of the references cited by the Examiner. By analogy to *Zierden*, Appellants submit that the following points provide the basis for the patentability of Appellants' claimed invention:

> 1. Zhang, or Weisburg, or Zhang in combination with any of Gallant, McCoy, or Stein fail to disclose the problem of losing affinity particles during manipulation of the particles in affinity procedures carried out in the absence of detergent
>
> 2. Appellants' method of controlling particle loss during particle handling in affinity separation procedures provides significant improvement in yields (see Examples 1-6 of the

specification) and is different from other affinity methods in the references, such as using columns containing immobilized affinity particles (as in McCoy and Gallant)

3. There is no mention or suggestion in any of the references or combinations thereof cited by the Examiner of how to prevent affinity particle loss during manipulations in an affinity procedure using Appellants' claimed method. (Detergent is present in the references only by way of examples of buffers used to achieve cell lysis, nucleic acid hybridization, removal of non-specifically bound artifacts, solubilization of fats, and preparation of standard chromatography columns for protein purification.)

It may be that some of the examples in the primary references (Zhang and Weisburg) may have enjoyed the benefit of practicing Appellants' claimed invention. It cannot be determined; the references are silent as to the characteristics of the affinity particles mentioned in passing in those references. In any event, it does not matter: Following the reasoning of the court in *Zierden*, a fair reading of the primary references or of the combination of references made by the Examiner indicates that such a practice of the claimed invention is only an example of an accidental use, unrecognized by any of the cited references, without profit to the art, and therefore without legal significance as an anticipation of the claimed invention. 411 F.2d at 1329, 162 USPQ at 105. Following *Zierden*, references of accidental use of a claimed method are not sufficient to serve as the basis for a *prima facie* case of anticipation under 35 U.S.C. § 102 or of obviousness under 35 U.S.C. § 103. Accordingly, the rejections of the appealed claims based on Zhang, or Weisburg, or the combination of Zhang with any of McCoy, Gallant, or Stein, are clearly improper as a matter of law. As a result, all of the rejections of the final action (Paper No. 10) should be reversed by the Board.

CONCLUSION

For the reasons discussed herein, none of the citations relied on by the Examiner to finally reject the claims on appeal contains any teaching relevant to improving a method for affinity separation to reduce the loss of affinity particles during the steps of the separation, by employing detergent with the affinity particles in one or more of the method steps. Moreover, none of the references inherently teaches either the problem or the benefit of Appellants' claimed method for improving affinity separations.

Therefore:

I. Zhang does NOT describe the invention defined in appealed Claims 1-4, 9, 13-19, 23, 24, 31-35, 44-50, 54, 55, and 62-66 in the manner prohibited under 35 U.S.C. § 102(e), and the rejection of those claims over Zhang should be reversed.

II. Weisburg does NOT anticipate the invention defined in appealed Claims 1-4, 9, 13-17, 20, 33-35, 44-46, 48, 49, and 64-66 in the manner prohibited under 35 U.S.C. § 102(b), and the rejection of those claims over Weisburg should be reversed.

III. The invention as defined in appealed Claims 1-4, 9, 13-17, 20, 25, 26, 33-35, 44-49, 56, 57, and 64-66 would NOT have been obvious to a person of ordinary skill in the art in view of the teachings of Weisburg, and the rejection of those claims over Weisburg should be reversed.

IV. The invention as defined in appealed Claims 1-19, 23, 24, 31-50, 54, 55, and 62-66 would NOT have been obvious to a person of ordinary skill in the art in view of the combined teachings of Zhang in view of McCoy, and the rejection of those claims over Zhang combined with McCoy should be reversed.

V. The invention as defined in Claims 1-4, 9, 13-19, 21, 23, 29-35, 44-50, 52, 54, 55, and 60-66 would NOT have been obvious to a person of ordinary skill in the art in view of the combined teachings of Zhang in view of Gallant, and the rejection of those claims over Zhang combined with Gallant should be reversed.

VI. The invention as defined in appealed Claims 1-4, 9, 13-19, 22, 27-29, 31-35, 44-50, 52, 53-55, and 58-66 would NOT have been obvious to a person of ordinary skill in the art in view of the combined teachings of Zhang in view of Stein, and the rejection of those claims over the combination of Zhang and Stein should be reversed.

Reversal of the final rejections and allowance of the claims on appeal are respectfully requested.

Respectfully submitted,

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Leon R. Yankwich

APPENDIX: Claims On Appeal in U.S. Serial No. 09/353,407

1. A method of separating particles from a solution while minimizing particle loss comprising the steps of:

   (a) combining a solution and a finely divided particulate matrix material, where said matrix material is insoluble in said solution, in the presence of a detergent;

   (b) collecting the particles of the particulate matrix material; and

   (c) separating the supernatant from the particulate matrix material.

2. In a method for isolating a molecule from a sample in a vessel using affinity particles, comprising the steps of:

   (a) combining the sample containing a molecule of interest with affinity particles suitable for binding said molecule, said affinity particles being insoluble in the sample;

   (b) collecting the affinity particles;

   (c) separating the affinity particles from the unbound remainder of the sample;

   (d) optionally, resuspending the affinity particles in a solution;

   (e) optionally, eluting said molecule from the affinity particles, followed by separating the affinity particles from said eluted molecule;

   the improvement wherein at least one of steps (a), (b), (c), (d) if present, and (e) if present is performed in the presence of detergent wherein the amount of detergent is sufficient to reduce loss of particles during any separation step, in comparison to the same method performed in the absence of detergent.

3. The method according to Claim 1 or 2, wherein the combining step (a) is carried out in the absence of detergent, but detergent is added prior to the separation step (b).

4. The method according to Claim 2, wherein said molecule of interest is selected from the group consisting of peptides, polypeptides, proteins, nucleotides, nucleic acids, carbohydrates, lipids, complexes, and organic molecules.

5. The method according to Claim 2, wherein said molecule is a fusion protein or peptide.

6. The method according to Claim 5, wherein said fusion protein is a protein or peptide fused to a metal chelating group.

7. The method according to Claim 6, wherein said metal chelating group is two or more histidine residues.

8. The method according to Claim 6, wherein said metal chelating group is six consecutive histidine residues.

9. The method according to Claim 2, wherein said molecule is a nucleic acid of interest.

10. The method according to Claim 9, wherein said nucleic acid of interest is fused to a metal chelating group.

11. The method according to Claim 10, wherein said metal chelating group is two or more histidine residues.

12. The method according to Claim 11, wherein said metal chelating group is six consecutive histidine residues.

13. The method according to Claim 1 or 2, wherein said particles are selected from the group consisting of ferromagnetic beads, superparamagnetic beads, and combinations thereof.

14. The method according to Claim 1 or 2, wherein said particles are composed of materials selected from the group consisting of agarose, silica, nitrocellulose, cellulose, acrylamide, latex, polystyrene, polyacrylate, polymethacrylate, polyethylene polymers such as polyvinyl alcohol, glass particles, silicates including calcium, magnesium and aluminum silicates, metal oxides including titanium oxides, and tin oxides, apatites, and combinations thereof.

15. The method according to Claim 14, wherein said particles are coated with an affinity ligand selected from the group consisting of antibodies for a particular antigen, antigens for a particular antibody, antibodies recognizing a class of molecules, streptavidin, streptavidin-tagged fusion proteins, biotin, biotin-tagged fusion proteins, glutathione,

cellulose, amylose, ion exchange groups, hydrophobic interaction groups, oligo-dT, nucleic acid polynucleotides complementary to a nucleic acid of interest, binding molecules for cell-surface markers, phage ligands, antibodies recognizing cell or phage surface antigens, and polypeptides, nucleotides or small molecules capable of affinity interactions with a binding partner selected from the group consisting of another protein, DNA, RNA, and small molecules.

16. The method according to Claim 1 or 2, wherein said detergent, where present, is at a concentration of from about 0.0005% to 2.0% (v/v).

17. The method according to Claim 16, wherein said detergent is selected from a group consisting of nonionic detergents, anionic detergents, zwitterionic detergents, cationic detergents, and combinations thereof.

18. The method according to Claim 17, wherein said nonionic detergent is selected from the group consisting of polyoxyethylene (10) cetyl alcohol, polyoxyethylene (20) cetyl alcohol, polyoxyethylene (23) lauryl alcohol, polyoxyethylene (4-5) *p-t*-octyl phenol, polyoxyethylene (7-8) *p-t*-octyl phenol, polyoxyethylene (9) *p-t*-octyl phenol, polyoxyethylene (9-10) *p-t*-octyl phenol, polyoxyethylene (9-10) nonylphenol, polyoxyethylene (20) sorbitol monolaurate, polyoxyethylene (20) sorbitol monopalmitate, polyoxyethylene (20) sorbitol monooleate, octyl-β-glucoside, APO-10, APO-12, cyclohexyl-*n*-ethyl-β-D-maltoside, cyclohexyl-*n*-hexyl-β-D-maltoside, cyclohexyl-*n*-methyl-β-maltoside, *n*-decanoylsucrose, *n*-decyl-β-D-glucopyranoside, *n*-decyl-β-maltopyranoside, *n*-decyl-β-D-thiomaltoside, *n*-dodecanoyl sucrose, and heptane-1,2,3-triol, and combinations thereof.

19. The method according to Claim 17, wherein said nonionic detergent is polyoxyehtylene (20) sorbitol monolaurate.

20. The method according to Claim 17, wherein said anionic detergent is selected from the group consisting of sodium dodecyl sulfate (SDS), sarkosyl, and combinations thereof.

21. The method Claim 17, wherein said zwitterionic detergent is 3-[(cholamido-propyl)-dimethyl-ammonio]-1-propanesulfonate.

22. The method according to Claim 17, wherein said cationic detergent dodecyl-trimethyl ammonium chloride.

23. The method according to Claim 1 or 2, wherein the detergent, where present, is a nonionic detergent at a concentration of at least about 0.005% (v/v).

24. The method according to Claim 1 or 2, wherein the detergent, where present, is a nonionic detergent at a concentration not exceeding about 2% (v/v).

25. The method according to Claim 1 or 2, wherein the detergent, where present, is an anionic detergent at a concentration of at least about 0.05% (v/v).

26. The method according to Claim 1 or 2, wherein the detergent, where present, is an anionic detergent at a concentration not exceeding about 1% (v/v).

27. The method according to Claim 1 or 2, wherein the detergent, where present, is a a cationic detergent at a concentration of at least about 0.5% (v/v).

28. The method according to Claim 1 or 2, wherein the detergent, where present, is a a cationic detergent at a concentration not exceeding about 1% (v/v).

29. The method according to Claim 1 or 2, wherein the detergent, where present, is a zwitterionic detergent at a concentration of at least about 0.01% (v/v).

30. The method according to Claim 1 or 2, wherein the detergent, where present, is a is a zwitterionic detergent at a concentration not exceeding about 2% (v/v).

31. The method according to Claim 1 or 2, wherein the molecule is a nucleic acid and the detergent is polyoxyethylene (20) sorbitol monolaurate at a concentration of at least about 0.005% (v/v).

32. The method according to Claim 1 or 2, wherein the molecule is a protein or peptide and the detergent is polyoxyethylene (20) sorbitol monolaurate at a concentration of at least about 0.005% (v/v).

33. A method of separating particles from a solution comprising the steps of:

(a) incubating a finely divided particulate matrix material in the presence of a detergent wherein the amount of detergent is sufficient to reduce loss of particles during any separation step;

(b) dispersing said particulate matrix material in a sample;

(c) collecting the particles of the particulate matrix material; and

(d) separating the supernatant from the particulate matrix material.

34. In a method for isolating a molecule of interest from a sample in a vessel using affinity particles, comprising the steps of:

(a) providing a multiplicity of affinity particles having a binding affinity for said molecule of interest;

(b) combining the sample containing the molecule of interest with affinity particles suitable for binding said molecule of interest, said affinity particles being insoluble in the sample;

(c) collecting the affinity particles;

(d) separating the affinity particles from the unbound remainder of the sample;

(e) optionally, resuspending the affinity particles in a solution;

(f) optionally, eluting said molecule of interest from the affinity particles, followed by separating the affinity particles from said eluted molecule;

the improvement wherein the affinity particles in step (a) are incubated in the presence of a detergent in an amount sufficient to reduce loss of particles during any subsequent separation step, in comparison to the same method performed in the absence of detergent, and wherein any of the steps (b), (c), (d), (e) if present, and (f) if present may optionally be also performed in the presence of detergent.

35. The method according to Claim 34, wherein said molecule of interest is selected from the group consisting of peptides, polypeptides, proteins, nucleotides, nucleic acids, carbohydrates, lipids, complexes, and organic molecules.

36. The method according to Claim 34, wherein said molecule is a fusion protein or peptide.

37. The method according to Claim 36, wherein said fusion protein is a protein or peptide fused to a metal chelating group.

38. The method according to Claim 37, wherein said metal chelating group is two or more histidine residues.

39. The method according to Claim 37, wherein said metal chelating group is six consecutive histidine residues.

40. The method according to Claim 34, wherein said molecule is a nucleic acid of interest.

41. The method according to Claim 40, wherein said nucleic acid of interest is fused to a metal chelating group.

42. The method according to Claim 41, wherein said metal chelating group is two or more histidine residues.

43. The method according to Claim 41, wherein said metal chelating group is six consecutive histidine residues.

44. The method according to Claim 33 or 34, wherein said particles are selected from the group consisting of ferromagnetic beads, superparamagnetic beads, and combinations thereof.

45. The method according to Claim 33 or 34, wherein said particles are composed of materials selected from the group consisting of agarose, silica, nitrocellulose, cellulose, acrylamide, latex, polystyrene, polyacrylate, polymethacrylate, polyethylene polymers such as polyvinyl alcohol, glass particles, silicates including calcium, magnesium and aluminum silicates, metal oxides including titanium oxides, and tin oxides, apatites, combinations thereof.

46. The method according to Claim 45, wherein said particles are coated with an affinity ligand selected from the group consisting of antibodies for a particular antigen, antigens for a particular antibody, antibodies recognizing a class of molecules, streptavidin, streptavidin-tagged fusion proteins, biotin, biotin-tagged fusion proteins, glutathione, cellulose, amylose, ion exchange groups, hydrophobic interaction groups, oligo-dT, nucleic acid polynucleotides complementary to a nucleic acid of interest, binding molecules for cell-surface markers, phage ligands, antibodies recognizing cell or phage surface antigens, and polypeptides capable of affinity interactions with a binding partner selected from the group consisting of another protein, DNA, RNA, and small molecules.

47. The method according to Claim 33 or 34, wherein said detergent, where present, is at a concentration of from about 0.0005% to 2.0% (v/v).

48. The method according to Claim 47, wherein said detergent is selected from a group consisting of nonionic detergents, anionic detergents, zwitterionic detergents, cationic detergents, and combinations thereof.

49. The method according to Claim 48, wherein said nonionic detergent is selected from the group consisting of polyoxyethylene (10) cetyl alcohol, polyoxyethylene (20) cetyl alcohol, polyoxyethylene (23) lauryl alcohol, polyoxyethylene (4-5) *p-t*-octyl phenol, polyoxyethylene (7-8) *p-t*-octyl phenol, polyoxyethylene (9) *p-t*-octyl phenol, polyoxyethylene (9-10) *p-t*-octyl phenol, polyoxyethylene (9-10) nonylphenol, polyoxyethylene (20) sorbitol monolaurate, polyoxyethylene (20) sorbitol monopalmitate, polyoxyethylene (20) sorbitol monooleate, octyl-β-glucoside, APO-10, APO-12, cyclohexyl-*n*-ethyl-β-D-maltoside, cyclohexyl-*n*-hexyl-β-D-maltoside, cyclohexyl-*n*-methyl-β-maltoside, *n*-decanoylsucrose, *n*-decyl-β-D-glucopyranoside, *n*-decyl-β-maltopyranoside, *n*-decyl-β-D-thiomaltoside, *n*-dodecanoyl sucrose, and heptane-1,2,3-triol, and combinations thereof.

50. The method according to Claim 49, wherein said nonionic detergent is polyoxyethylene (20) sorbitol monolaurate.

51. The method according to Claim 48, wherein said anionic detergent is selected from the group consisting of sodium dodecyl sulfate (SDS), sarkosyl, and combinations thereof.

52. The method Claim 48, wherein said zwitterionic detergent is 3-[(cholamido-propyl)-dimethyl-ammonio]-1-propanesulfonate.

53. The method according to Claim 48, wherein said cationic detergent dodecyl-trimethyl ammonium chloride.

54. The method according to Claim 33 or 34, wherein the detergent, where present, is a nonionic detergent at a concentration of at least about 0.005% (v/v).

55. The method according to Claim 33 or 34, wherein the detergent, where present, is a nonionic detergent at a concentration not exceeding about 2% (v/v).

56. The method according to Claim 33 or 34, wherein the detergent, where present, is an anionic detergent at a concentration of at least about 0.05% (v/v).

57. The method according to Claim 33 or 34, wherein the detergent, where present, is an anionic detergent at a concentration not exceeding about 1% (v/v).

58. The method according to Claim 33 or 34, wherein the detergent, where present, is a a cationic detergent at a concentration of at least about 0.5% (v/v).

59. The method according to Claim 33 or 34, wherein the detergent, where present, is a a cationic detergent at a concentration not exceeding about 1% (v/v).

60. The method according to Claim 33 or 34, wherein the detergent, where present, is a zwitterionic detergent at a concentration of at least about 0.01% (v/v).

61. The method according to Claim 33 or 34, wherein the detergent, where present, is a is a zwitterionic detergent at a concentration not exceeding about 2% (v/v).

62. The method according to Claim 33 or 34, wherein the molecule is a nucleic acid and the detergent is polyoxyethylene (20) sorbitol monolaurate at a concentration of at least about 0.005% (v/v).

63. The method according to Claim 33 or 34, wherein the molecule is a protein or peptide and the detergent is polyoxyehtylene (20) sorbitol monolaurate at a concentration of at least about 0.005% (v/v).

64. In a method for isolating a molecule from a sample in a vessel using magnetic affinity particles, comprising the steps of:

(a) combining the sample containing a molecule of interest with magnetic affinity particles suitable for binding said molecule, said magnetic affinity particles being insoluble in the sample;

(b) applying a magnetic field to the vessel so as to attract and immobilize the magnetic affinity particles;

(c) separating the unimmobilized remainder of the sample from the immobilized magnetic affinity particles;

(d) optionally, resuspending the magnetic affinity particles in a solution;

(e) optionally, eluting said molecule from the affinity particles, followed by separating the affinity particles from said eluted molecule;

the improvement wherein at least one of steps (a), (b), (c), (d) if present, and (e) if present is performed in the presence of detergent wherein the amount of detergent is sufficient to reduce loss of particles during any subsequent separation step, in comparison to the same method performed in the absence of detergent.

65. The method according to Claim 64, wherein the combining step (a) is carried out in the absence of detergent, but detergent is added prior to the application of a magnetic field in accordance with step (b).

66. In a method for isolating a molecule of interest from a sample in a vessel, comprising the steps of:

(a) providing a multiplicity of magnetic affinity particles suitable for binding said molecule of interest;

(b) combining the sample containing a molecule of interest with said affinity particles suitable for binding said molecule, said affinity particles being insoluble in the sample;

(c) immobilizing the magnetic affinity particles by applying a magnetic field to said vessel;

(d) separating the remainder of the sample from the immobilized magnetic affinity particles;

(e) optionally, resuspending the affinity particles in a solution;

(f) optionally, eluting said molecule from the affinity particles, followed by separating the affinity particles from said eluted molecule;

the improvement wherein the affinity particles suitable for binding said molecule of interest in step (a) are incubated in the presence of a detergent in an amount sufficient to reduce loss of affinity particles during any subsequent separation step, in comparison to the same method performed in the absence of detergent; and

wherein any of the steps (b), (c), (d), (e) if present, and (f) if present may optionally be also performed in the presence of detergent.

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Biochemistry

Colony hybridization: A method for the isolation of cloned DNAs that contain a specific gene

(*Drosophila melanogaster* DNA/recombinant DNA molecules/plasmids/18–28S rRNA genes/autoradiography)

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ABSTRACT A method has been developed whereby a very large number of colonies of *Escherichia coli* carrying different hybrid plasmids can be rapidly screened to determine which hybrid plasmids contain a specified DNA sequence or genes. The colonies to be screened are formed on nitrocellulose filters, and, after a reference set of these colonies has been prepared by replica plating, are lysed and their DNA is denatured and fixed to the filter *in situ*. The resulting DNA-prints of the colonies are then hybridized to a radioactive RNA that defines the sequence or gene of interest, and the result of this hybridization is assayed by autoradiography. Colonies whose DNA-prints exhibit hybridization can then be picked from the reference plate. We have used this method to isolate clones of ColE1 hybrid plasmids that contain *Drosophila melanogaster* genes for 18 and 28S rRNAs. In principle, the method can be used to isolate any gene whose base sequence is represented in an available RNA.

Segments of DNA from *Drosophila melanogaster* chromosomes (Dm segments) can be isolated by cloning hybrid DNA molecules that consist of a Dm segment inserted into the circular DNA of an *Escherichia coli* plasmid. We have previously reported on the use of such cloned segments in the analysis of DNA sequence arrangements in the *D. melanogaster* genome (1–3). However, that analysis has been limited by our inability to isolate cloned Dm segments that contain a specified DNA sequence or gene. In this article we describe a procedure that permits the isolation of such specific Dm segments, and which can be extended to DNA segments from any organism.

Experimental Plan. Consider an experiment in which the Dm segments in a random set are individually inserted into a given *E. coli* plasmid. Transformation of *E. coli* by these hybrid plasmids to a phenotype conferred by genes in the parental plasmid will yield colonies that individually contain a single cloned Dm segment (1–3). If these segments are randomly distributed and exhibit a mean length of 10,000 base pairs, or 10 kb, then we expect that about one colony in 16,000 will contain a particular nonrepetitive *D. melanogaster* DNA sequence the length of a typical structural gene, i.e., 1–2 kb. Hence, the goal is to devise a screening procedure whereby one can rapidly determine which colony in thousands contains such a sequence.

The screening procedure that we have developed is designed to detect sequences that can hybridize with a given radioactive RNA. In this procedure the colonies to be screened are first grown on nitrocellulose filters that have been placed on the surface of agar petri plates prior to inoculation. A reference set of these colonies is then obtained by replica plating (4) to additional agar plates that are stored at 2–4°C. The colonies on the filter are lysed and their DNAs are denatured and fixed to the filter *in situ* to form a "DNA-print" of each colony. The defining, labeled RNA is hybridized to this DNA and the result of the hybridization is monitored by autoradiography on x-ray film. The colony whose DNA-print exhibits hybridization with the defining RNA can then be picked from the reference set.

The characteristics of this procedure and its application to the isolation of hybrid plasmids containing the *D. melanogaster* genes for '18' and '28'S rRNAs are described in this paper.

Abbreviations: kb (kilobases), 1000 bases or base pairs in single- or double-stranded nucleic acids, respectively; Dm, a segment of *Drosophila melanogaster* DNA; cDm and pDm, hybrid plasmids consisting of a Dm segment inserted into ColE1 and pSC101 DNAs, respectively; SSC = 0.15 M NaCl, 0.015 M sodium citrate; cRNA, RNA complementary to DNA; rDNA, DNA coding for ribosomal RNA.

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MATERIALS AND METHODS

Bacteria. *E. coli* K12 strains HB101, HB101 [pDm103], and C600 [pSC101] are those used previously (plasmids are indicated in brackets) (3). Strain W3110 has been described (5), and W3110 [ColE1] was obtained from D. R. Helinski.

DNAs, Complementary RNAs (cRNAs), and Enzymes. pDm103 (3) and ColE1 (6) DNAs were generously provided by D. M. Glover and D. J. Finnegan, respectively, and were prepared from HB101 [pDm103] and W3110 [ColE1] according to the indicated references, except that the ColE1 was amplified by overnight incubation of W3110 [ColE1] in the presence of chloramphenicol (7) prior to lysis. ^{32}P- and ^{3}H-labeled cRNAs were transcribed *in vitro* from these DNAs with *E. coli* RNA polymerase (8), as described by Wensink *et al.* (1). The RNA polymerase was prepared according to the indicated reference, and was the generous gift of W. Wickner. Pancreatic ribonuclease and proteinase K were obtained from Worthington Biochemical Corp. and E. Merck Laboratories, respectively.

Colony hybridization

Formation of the Filter and Reference Sets of Colonies. Colonies are formed on Millipore HA filters (0.45 μm pores) that have been washed three times in boiling H_2O (1 min per wash), placed between sheets of absorbant paper, autoclaved at 120° for 10 min, and dried for 10 min in the autoclave. The filter is then placed on an L-agar petri plate (1) and the desired bacteria are transferred to the filter surface either by spreading or using sterile toothpicks to obtain ≤ 7 colonies per cm^2 after incubation of the filter-plate at 37°. The reference set is produced by replica plating of the colonies that develop on the filter to L-agar plates and is stored at 2–4°.

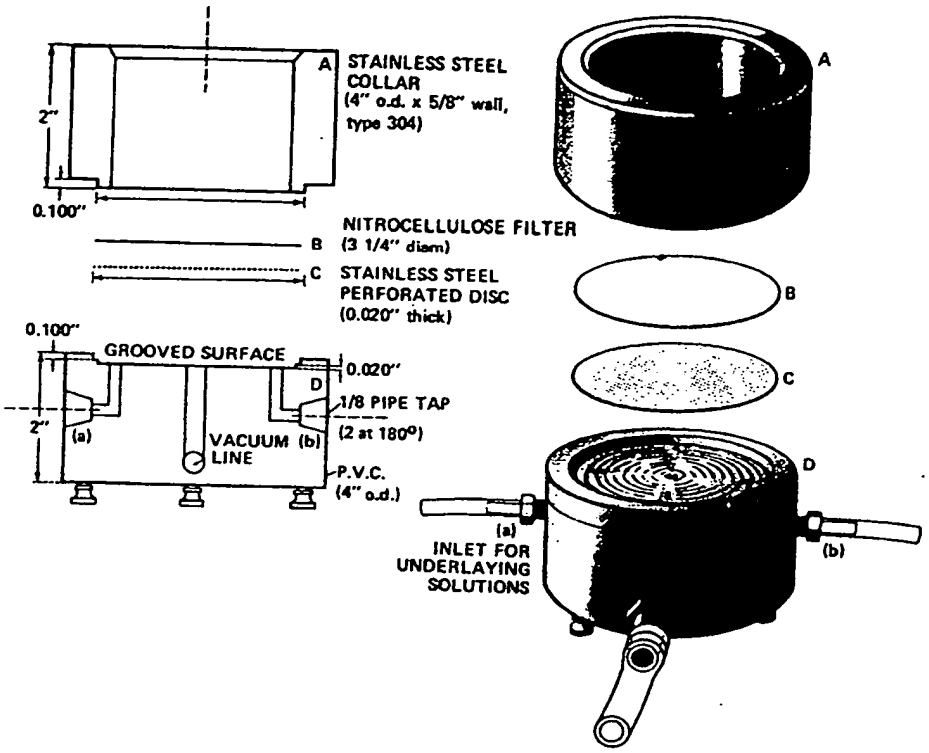

FIG. 1. Apparatus for treatment of colonies on filters. To wet the underside of the filter, solutions are introduced through ports (a) or (b), while the tube connected to the vacuum port is clamped off. Solutions are removed through the vacuum port which is connected to a water aspirator. Other procedures are described in the text. ", inches (2.54 cm); o.d., outside diameter; P.V.C., polyvinyl chloride.

Lysis, DNA Denaturation, and Fixation. To prevent movement of the bacteria or DNA from their colonial sites during lysis, denaturation and fixation, the solutions used to effect these reactions are applied to the underside of the filter and allowed to diffuse into the colony. The apparatus shown in Fig. 1 has been designed for this purpose. The filter is lifted from the agar plate and placed on the perforated disc that is set in a plastic cylinder which has ports cut into it to introduce solutions sequentially to the underside of the filter and to apply vacuum. Unless otherwise indicated, all operations are carried out at room temperature (20–25°).

Lysis and DNA denaturation are effected by introducing 0.5 N NaOH beneath the filter until it barely floats. After 7 min the NaOH is slowly removed with a minimum of vacuum, and replaced by 1.0 M Tris·HCl (pH 7.4) for 1 min. This solution is replaced with the same buffer, after which the pH of the solution in contact with the filter should be approximately neutral. The last wash is replaced by 1.5 M NaCl, 0.5 M Tris·HCl (pH 7.4), which is removed after 5 min. The stainless steel collar is then placed over the filter, and full vacuum is applied for approximately 2 min until the colonial residues assume a dry appearance. At this point there is less danger of movement from the colonial site and the remaining solutions can be layered on the upper side of the filter.

A 2 mg/ml solution of proteinase K in 1 × SSC (0.15 M NaCl, 0.015 M sodium citrate) is added to just cover the filter. After 15 min, it is removed by vacuum filtration, and 95% ethanol (1 ml/cm^2 of filter) is similarly passed through the filter. After five washes effected by passing chloroform through the filter (2 ml/cm^2 per wash), the filter is removed from the apparatus, dipped into 0.3 M NaCl to remove loose cellular debris, and baked at 80° *in vacuo* for 2 hr.

Hybridization and ^{32}P-Autoradiography or ^{3}H-Fluorography. The dry filter is moistened with a 5 × SSC, 50% formamide solution containing the labeled RNA, using 10–15 μl/cm^2 of filter. The filter is covered with mineral oil, incubated for 16 hr at 37° to allow hybridization, and then washed for 10 min in a beaker containing chloroform that is gently agitated on a shaking platform. Two more identical chloroform washes are followed by 10 min washes in 6 × SSC, 2 × SSC, and 2 × SSC containing 20 μg/ml of pancreatic ribonuclease. If the RNA is ^{32}P-labeled, the filter is blotted to remove excess liquid, covered with Saran Wrap, and placed under Kodak RPS/54 x-ray film for autoradiography. If the RNA is ^{3}H-labeled, the filter is dried for 30 min at 80° *in vacuo*, and 40 μl of 7% 2,5-diphenyloxazole (PPO) in ether is applied per cm^2 of filter. The dry filter is then placed under x-ray film for fluorography at −82° (9).

RESULTS

Colony hybridization distinguishes between [ColE1]$^+$ and [ColE1]$^-$ bacteria

We have turned increasingly toward the use of the colicinogenic plasmid, ColE1, as a cloning vector because one can obtain much higher cellular concentrations of its hybrids (7) than is the case for the tetracycline resistance plasmid, pSC101, which we used previously (1–3). The first test system for colony hybridization therefore consisted of ^{32}P-labeled cRNA made by transcription of ColE1 DNA *in vitro* with *E. coli* RNA polymerase, and *E. coli* containing or not containing ColE1, i.e., [ColE1]$^+$ or [ColE1]$^-$ bacteria.

Fig. 2A shows the autoradiographic response obtained after hybridization of [^{32}P]cRNA to the DNA-prints of [ColE1]$^+$ and [ColE1]$^-$ colonies formed on nitrocellulose fil-

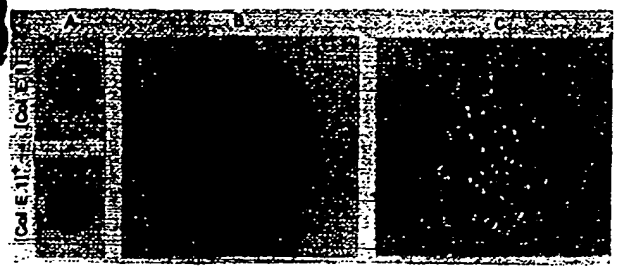

FIG. 2. Hybridization of ColE1 cRNA to [ColE1]$^-$ and [ColE1]$^+$ colonies. The procedures for colony hybridization, autoradiography, and fluorography are described in *Materials and Methods*, as are the W3310 and W3110 [ColE1] *E. coli* strains used to form the [ColE1]$^-$ and [ColE1]$^+$ colonies, respectively. (A) 1×10^5 cpm of [^{32}P]cRNA (5×10^7 cpm/μg) were applied to each 13-mm filter (area = 1.3 cm^2) in a 20 μl volume. After hybridization, the DNA-prints of [ColE1]$^+$ colonies contained an average of 1.8×10^2 cpm per colony, which is 30-fold greater than the background radiation from an equivalent area on the filter. Exposure time = 45 min. (B) A mixture of [ColE1]$^+$ and [ColE1]$^-$ bacteria in a 1:100 ratio was spread on a 47-mm filter (area = 17.3 cm^2) to obtain a total of 1 to 2×10^2 colonies per filter; 5×10^5 cpm of [^{32}P]cRNA (3×10^7 cpm/μg) in 250 μl were applied to the filter. Exposure time = 4 hr. (C) A 1:1 mixture of [ColE1]$^+$ and [ColE1]$^-$ bacteria was spread on a 47-mm filter to obtain a total of 93 colonies, of which 52 gave the A$^+$ response seen in the figure; 1×10^6 cpm of [^{3}H]cRNA (2×10^7 cpm/μg) in 200 μl were applied to the filter. Exposure time = 24 hr.

ters. The positive response given by the [ColE1]$^+$ colonies is abbreviated by A$^+$ and the negative response of [ColE1]$^-$ colonies by A$^-$. Colonies obtained by spreading mixtures of [ColE1]$^+$ and [ColE1]$^-$ bacteria in different ratios gave the expected frequencies of A$^+$ and A$^-$ responses. Fig. 2B shows the result obtained when [ColE1]$^+$/[ColE1]$^-$ = 1/100.

A more precise measure of the specificity of colony hybridization of mixtures is given by the following experiment in which a 1:1 mixture of [ColE1]$^+$ and [ColE1]$^-$ bacteria was spread on a filter to yield 31 colonies. Hybridization and autoradiography revealed that 16 were A$^+$ and 15 A$^-$. Bacteria from each of the corresponding colonies on the agar replica plate were then tested for colicin production according to an overlay technique described by Finnegan and Willets (10). All 16 A$^+$ colonies were colicin-positive (i.e., [ColE1]$^+$); all 15 A$^-$ colonies were colicin-negative and therefore presumed to be [ColE1]$^-$.

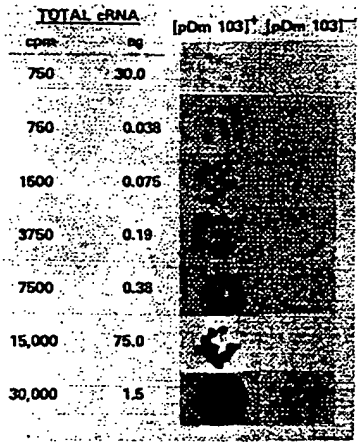

FIG. 3. Hybridization of different amounts of pDm103 [^{32}P]cRNA to [pDm103]$^+$ and [pDm103]$^-$ colonies. Colonies were obtained by transferring HB101 [pDm103] or HB101 bacteria, respectively, to 13-mm filters with toothpicks. In the experiments where $\leq$1.5 ng of cRNA were applied per filter, the specific activity = 2×10^7 cpm/μg. The lower specific activities used for the other two experiments were obtained by mixing this cRNA with unlabeled pDm103 cRNA. The weak response observed for [pDm103]$^-$ colonies could result either from *E. coli* DNA impurities in the pDm103 DNA preparations used to prepare the [^{32}P]cRNA, or from some similarity of sequence in pDm103 and *E. coli* DNAs.

Fig. 2A and B show that the position of A$^-$ colonies can be detected on the autoradiograph because of the higher background radiation from the filter itself. While this background radiation is convenient for the direct visualization of A$^-$ colonies and is not critical to the observation of the A$^+$ response obtained with cRNAs, it may become an important factor with other RNAs if they give a weaker A$^+$ response. Our observations indicate that the level of this background varies with the preparation of labeled RNA and, possibly, with the batch of filters, but we have not examined such factors in detail.

Fig. 2C shows that the colony hybridization procedure can be adapted to ^{3}H-labeled cRNA by impregnating the filter with 2,5-diphenyloxazole after hybridization and prior to placement on the x-ray film (*Materials and Methods*). Of the 93 colonies obtained by spreading a 1:1 mixture of [ColE1]$^+$ and [ColE1]$^-$ bacteria, 52 were A$^+$ and 41 A$^-$. We estimate from the extent of the A$^+$ response that this ^{3}H-fluorography is about one-twentieth as efficient as the ^{32}P-autoradiography.

The autoradiographic response is proportional to the total radioactivity of the applied cRNA and insensitive to its specific activity

We next examined the dependence of the A$^+$ response on the total and the specific radioactivity of the applied cRNA. In this case, the ^{32}P-labeled cRNA was transcribed *in vitro* from a hybrid plasmid called pDm103, and hybridized to DNA-prints of colonies that either contained this hybrid, [pDm103]$^+$, or did not, [pDm103]$^-$. The pDm103 hybrid was formed between pSC101 plasmid DNA (9 kb) and a segment of *D. melanogaster* DNA (Dm103; 17 kb) that contains the gene for '18' and '28'S rRNAs (3).

Fig. 3 shows that the autoradiographic response obtained when pDm103 [^{32}P]cDNA was hybridized to 13-mm filters containing [pDm103]$^+$ colonies is roughly proportional to the total radioactivity. It is clearly insensitive to the mass of cRNA containing that radioactivity, i.e., to its specific activity. For example, the response to 750 cpm of [^{32}P]cRNA is approximately the same whether contained in 0.038 ng or in 30 ng. Similarly the response to 15,000 cpm contained in 75 ng is intermediate between that to 7,500 cpm and 30,000 cpm, although the last two samples contained only 0.38 and 1.5 ng, respectively. This would suggest that the RNA·DNA hybridization is occurring under conditions of DNA excess even when 75 ng of pDm103 cRNA are applied per 13 mm filter. However, we have calculated that there is only some 2 ng of pDm103 DNA per colony [i.e., (2×10^7 cells per colony) $\times$ (4 pDm103 per cell) 2.9×10^{-8} ng DNA per pDm103]. This value is based on our observation of 2×10^7 cells per 1 mm colony and the presence of 4 pDm103 per cell in liquid culture (3). Evidently only a small fraction of the applied cRNA can react with the DNA-prints on the filter even though the reaction is occurring ostensibly in DNA excess. A similar result was observed when ColE1 cRNA was hybridized to [ColE1]$^+$ colonies (legend, Fig 2A). Of 2 ng cRNA applied to each filter only 0.004 ng (i.e., 0.2%) hybridized per [ColE1]$^+$ colony. A 1 mm [ColE1]$^+$ colony is estimated to contain 3–4 ng of ColE1 DNA.

A simple explanation of these results is obtained if one assumes that most or all of the cRNA in the small fraction of the RNA solution which wets a DNA-print will hybridize, and that the remainder of the cRNA will not hybridize at a significant rate, due perhaps to its slow diffusion through the nitrocellulose, or because of other barriers. Thus a DNA-print from a 1-mm colony, which occupies 0.6% of the area of a 13-mm filter, would be expected to hybridize ≤0.6% of the applied RNA, an expectation that is compatible with the 0.2% observed. For a given ratio of colony to filter area, the fraction of applied cRNA that hybridizes to a DNA-print, in conditions of local DNA excess, would therefore be constant and independent of the total applied cRNA over a wide range of values.

Colony hybridization with cRNA to pDm103 provides a screen for cDm plasmids containing *D. melanogaster* rDNA

Hybrid plasmids consisting of a Dm segment inserted into ColE1 DNA are called cDm plasmids, as distinguished from pDm plasmids where the Dm segment has been inserted into pSC101. In this section we describe two applications of colony hybridization that result in the isolation of cDm plasmids that contain DNA from the repeating gene-spacer units for 18–28S rRNAs (i.e., rDNA) in *D. melanogaster* (3). In the first application, [^{32}P]cRNA to pDm103 was used to isolate clones of cDm103 plasmids; i.e., plasmids in which the Dm103 segment is inserted into ColE1 DNA at its single *Eco*RI endonuclease cleavage site (7). In the second application, the same [^{32}P]cRNA was used to screen a large set of random cDm clones for rDNA. cRNA formed by transcription of the entire pDm103 DNA can be used for these purposes since we have demonstrated that pSC101 and ColE1 sequences do not interact to give a significant A^+ response (data not shown).

Cleavage of circular pDm103 DNA with the *Eco*RI restriction endonuclease yields intact Dm103 segments and linear pSC101 DNA (3). In cooperation with D. M. Glover, we treated a mixture of *Eco*RI-cleaved pDm103 and ColE1 DNAs with *E. coli* ligase under previously described conditions (3), and then transformed colicin-sensitive *E. coli* to colicin E1 immunity with this mixture of ligated DNAs (11). Since the *Eco*RI termini of the linear ColE1, pSC101, and Dm103 molecules can be randomly joined by the ligase, any of the following circular products of this ligation may be present in the colonies of transformants: (*i*) recyclized ColE1 (monomers, dimers, etc), (*ii*) molecules containing one ColE1 and one pSC101 segment [abbreviated by $(c)_1(p)_1$], (*iii*) $(c)_1(Dm103)_1$ molecules, i.e., the desired cDm103 plasmids, or (*iv*) rarer more complex combinations, such as $(c)_1(p)_1(Dm103)_1$, which contain one or more copies of ColE1.

Forty-eight of the transformants were screened for the presence of either pSC101 or Dm103 segments by colony hybridization with [^{32}P]cRNA to pDm103 (Fig. 4A), and for the presence of the pSC101 segment by testing for resistance to tetracycline. Of the eight A^+ transformants shown in Fig. 4A, six were tetracycline resistant and probably contain $(c)_1(p)_1$ plasmids. They were not examined further. The remaining two (indicated by 1 and 2 in Fig. 4A) were tetracycline sensitive, and were assumed to contain cDm103 plasmids; they were designated cDm103/1 and cDm103/2, respectively.

Proof of this assumption was obtained by electron microscopic examination of the plasmids isolated from the two

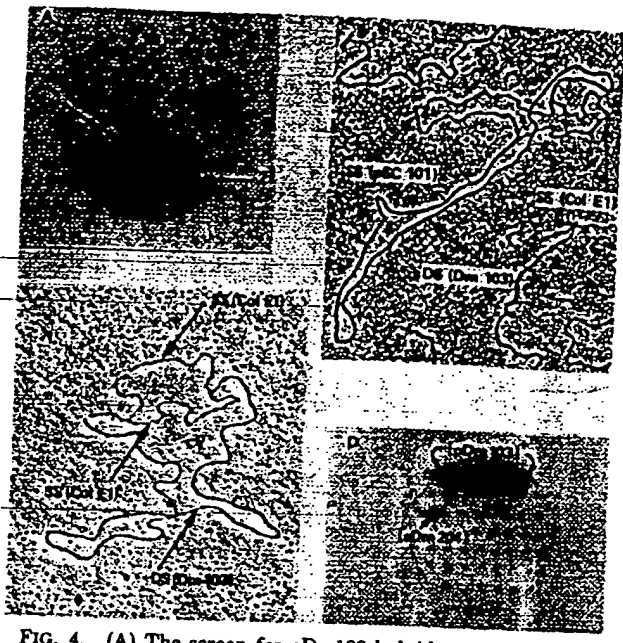

FIG. 4. (A) The screen for cDm103 hybrids. 5 μg of pDm103 DNA and 0.25 μg of ColE1 DNA were cleaved to completion with *Eco*RI endonuclease (in 0.120 ml of 0.1 M Tris-HCl, pH 7.5, 0.01 M $MgSO_4$), heated for 5 min at 65° to inactivate the enzyme and brought to 4°. The DNAs were then incubated at 14° with DNA ligase (14 μg/ml) in 0.1 M Tris-HCl, pH 7.5, as well as a reaction buffer consisting of 0.1 mM DPN, 1 mM EDTA, 10 mM $(NH_4)_2SO_4$, 10 mM $MgSO_4$ with 100 μg/ml of bovine serum albumin for 120 min in a total volume of 0.140 ml. The solution was then diluted 3-fold with the same reaction buffer and incubated for 36 hr at 14° in the presence of ligase (10 μg/ml). The ligated mixture of *Eco*RI-cleaved pDm103 and ColE1 DNAs (see *text*) was used to transform HB101 to colicin E1 immunity as described previously (11). Each of 48 transformants were transfered by toothpick to a 47-mm filter for colony hybridization (*Materials and Methods*), and to L-agar plates containing 15 μg of tetracycline per ml. 5×10^5 cpm of pDm103 [^{32}P]cRNA (2×10^7 cpm/μg) were used for the colony hybridization, which after a 6-hr exposure yielded the above autoradiograph. The colonies marked 1 and 2 contain cDm103/1 and cDm103/2 hybrids, respectively. (B) Electron micrograph of a pDm103-cDm103/2 heteroduplex. pDm103 and cDm103/2 circular DNAs were randomly nicked (broken in one strand) by x-rays. The procedures for denaturation and renaturation of these DNAs to form heteroduplexes, for spreading in 40% formamide prior to electron microscopy, and for measuring contour lengths have been described (1). pSC101 (9.2 kb; ref. 1) was used as an internal reference for double-stranded lengths (DS in the figure); no reference was used for single-stranded lengths (SS), as only the ratio of two SS-lengths is used in the analysis (see *text*). (C) Electron micrograph of a cDm103/1-cDm103/2 heteroduplex. The procedures are given in (B) above. See *text* for explanation. (D) The screen for cDm hybrids containing *D. melanogaster* rDNA. Hybrids between *Eco*RI-cut ColE1 and randomly broken Dm segments were formed as indicated in the *text*, and then used to transform HB101 to colicin E1 immunity as in (A) above. 300 independent transformants were transferred to six 47-mm filters, each of which contained six control colonies of HB101 [pDm103] at the top of the pattern. 5×10^5 cpm of pDm103 [^{32}P]cRNA (2×10^7 cpm/μg) was applied per filter for the colony hybridization. The autoradiograph in the figure resulted from one of the six filters after a 5-hr exposure, and shows one of the five rDNA hybrids (cDm204) identified by this screening procedure.

transformants, and of heteroduplexes formed between pDm103 and cDm103/2, and between cDm103/1 and cDm103/2. The mean lengths ±SD (n = 18) of cDm103/1 and cDm103/2 are 23.0 (±1.2) kb and 21.7 (±1.5) kb, respectively. The sum of the lengths of Dm103 (17 kb) and

ColE1 (6 kb; ref. 7) is 23 kb, in reasonable agreement with these values.

A heteroduplex formed between pDm103 and cDm103/2 is shown in Fig. 4B. It consists of a 17 kb double-stranded element whose ends are connected by each of two single-stranded elements that exhibit a length ratio of 1.5. This is the structure expected if cDm103/2 consists of a Dm103 segment inserted at the *Eco*RI cleavage site of ColE1; i.e., the double-stranded element represents the paired Dm103 segments of the two plasmid strands, and the larger and smaller single-stranded elements represent the pSC101 and ColE1 segments respectively (expected length ratio = 9 kb/6 kb = 1.5).

The heteroduplex formed between cDm103/1 and cDm103/2 consists of a 17 kb duplex whose ends are connected by two single-stranded elements of equal length (Fig. 4C). The simplest explanation of this structure is that the Dm103 segments were oppositely inserted into ColE1 during formation of cDm103/1 and cDm103/2. If the Dm103 segments in the single strands of two such oppositely oriented plasmids pair to create a 17 kb duplex element, then the two single-stranded ColE1 segments would contain identical rather than complementary base sequences, and could not pair.

The last experiment consists in screening hundreds of different $[cDm]^+$ colonies for rDNA. The $[cDm]^+$ colonies were obtained by transformation of colicin-sensitive *E. coli* to immunity with a heterogeneous population of cDm molecules constructed from *Eco*RI-cleaved ColE1 and random Dm segments (obtained by shear breakage) by the poly(dA)·poly(dT) joining method (1). These transformants were provided by D. J. Finnegan and G. Rubin. They were individually transferred by toothpick to six 47-mm nitrocellulose filters, each filter containing about 50 independent transformants. Colony hybridization with pDm103 [^{32}P]cRNA indicated no A^+ colonies on three filters, 1 A^+ colony on two filters, and 3 A^+ colonies on one filter. The autoradiograph of one of the two filters containing a single A^+ colony, cDm204, is given in Fig. 4D (the top row of A^+ colonies on the filter are $[pDm103]^+$ controls). When each of the 5 A^+ colonies was retested by repeating this colony hybridization on subclones, such subclones were consistently A^+.

Since pSC101 and ColE1 sequences do not interact to give an A^+ response, we presume that the cDm plasmids in these 5 A^+ colonies contain sequences present in Dm103; i.e., they contain rDNA from *D. melanogaster*. Indeed, D. M. Glover and R. L. White (personal communication) have shown recently that the 28 kb Dm segment in cDm204 contains the same arrangement of '18'–'28'S and spacer sequences as is found in Dm103.

DISCUSSION

In principle, colony hybridization of cloned hybrid plasmids can be used to isolate any gene, or other DNA segment, whose base sequence is represented in an available RNA. We used cRNA to pDm103 for the isolation of cDm plasmids containing rDNA. However, as we have observed that $[pDm103]^+$ colonies give an adequate A^+ response with ^{3}H-labeled '18' plus '28'S rRNAs isolated from *D. melanogaster* cell cultures (3), the isolation could have been accomplished with these rRNAs. For rRNA the genes are repeated hundreds of times per genome, and this is the reason that we were able to isolate several hybrids containing rDNA by screening only a few hundred colonies.

By contrast, we calculate that it would be necessary to screen approximately 50,000 hybrid clones to have a 95% chance of finding a hybrid containing a nonrepeated structural gene of typical length from *D. melanogaster*. From the data given in Fig. 3 and assuming 24-hr exposures, we estimate that this would require a total of approximately 4×10^6 cpm of [^{32}P]mRNA (specific activity $\geq 4 \times 10^5$ cpm/μg) applied to about one hundred thirty-five 82-mm filters. Thus a screen of this size is quite feasible. The isolation of nonrepeated genes from larger genomes would, of course, proportionately increase the number of colonies to be screened and hence the total required radioactivity.

An important advantage of colony hybridization is that it facilitates containment of any potentially hazardous hybrid plasmids that may be cloned in such large screening operations. By confining the reproductive state of the hybrid-clones to colonies, the probability of escape is reduced over that for liquid cultures because the number of bacteria per clone is generally smaller and aerosols or accidental spills are less likely. Furthermore the screening operation can be confined to small, controllable areas.

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where one sum, over i, is over all binding sites and the other, over j, is over all types of molecule in the bound solvent.

If there is only one type of molecule in the bound solvent, there will be only one term in the sum over j in equation (A4). Then, if the molecules in the bound solvent are water molecules, equation (A4) becomes identical with equation (24) in section 6(c) of the paper.

Like S in the special case considered in section 6(c), S and the S_j in the general case are related to the number and heights of peaks corresponding to binding sites that might be observed in a density map of the protein crystal structure. From equation (A4) it is clear that there is no simple relation between S and the total number of binding sites in a unit cell and any mean occupancy for the binding sites. Nevertheless, the value of S_j reveals the minimum number of binding sites at which molecules of type j will be found in the unit cell. If σ_{ij} for any site i is greater than 0 but less than 1, the number of molecules of type j in the bound solvent is less than the number of binding sites at which such molecules are located. If S_j and the total number of binding sites are known, the mean occupancy factor for molecules of type j can be calculated.

There may be many sites for which the sum of occupancy factors is less than 1. When these sites are not occupied by molecules contributing to the bound solvent, i.e. to the value of S, they are occupied by one or more of the following: (1) molecules contributing to the free solvent, perhaps because they do not have the proper orientation to contribute to the bound solvent, or (2) molecules contributing to the bound solvent but occupying neighboring, overlapping sites, or (3) movable side chains of the protein.

If D_c for a crystal with more than one type of molecule in the bound solvent is a linear function of D_s, S and v_p^s are independent of the composition of the free solvent (see section 3(a)). Like S, v_p^s is a function of the number and occupancies of binding sites. For the same reasons given in the conclusion of section 6(c), the independence of S and v_p^s from the composition of free solvent indicates that the number and occupancies of binding sites are nearly independent of that composition.

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Detection of Specific Sequences Among DNA Fragments Separated by Gel Electrophoresis

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This paper describes a method of transferring fragments of DNA from agarose gels to cellulose nitrate filters. The fragments can then be hybridized to radioactive RNA and hybrids detected by radioautography or fluorography. The method is illustrated by analyses of restriction fragments complementary to ribosomal RNAs from *Escherichia coli* and *Xenopus laevis*, and from several mammals.

1. Introduction

Since Smith and his colleagues (Smith & Wilcox, 1970; Kelly & Smith, 1970) showed that a restriction endonuclease from *Haemophilus influenzae* makes double-stranded breaks at specific sequences in DNA, this enzyme and others with similar properties have been used increasingly for studying the structure of DNA. Fragments produced by the enzymes can be separated with high resolution by electrophoresis in agarose or polyacrylamide gels. For studies of sequences in the DNA that are transcribed into RNA, it would clearly be helpful to have a method of detecting fragments in the gel that are complementary to a given RNA. This can be done by slicing the gel, eluting the DNA and hybridizing to RNA either in solution, or after binding the DNA to filters. The method is time consuming and inevitably leads to some loss in the resolving power of gel electrophoresis. This paper describes a method for transferring fragments of DNA from strips of agarose gel to strips of cellulose nitrate. After hybridization to radioactive RNA, the fragments in the DNA that contain transcribed sequences can be detected as sharp bands by radioautography or fluorography of the cellulose nitrate strip. The method has the advantages that it retains the high resolving power of the gel, it is economical of RNA and cellulose nitrate filters, and several electrophoretograms can be hybridized in one day. The main disadvantage is that fragments of 500 nucleotide pairs or less give low yields of hybrid and such fragments will be under-represented or even missing from the analysis.

2. Materials, Methods and Results

(a) *Restriction endonucleases*

EcoRI prepared according to the method of Yoshimuri (1971) was a gift of K. Murray. HaeIII prepared by a modification of the method of Roberts (unpublished data) was a gift of H. J. Cooke.

(b) *Gel electrophoresis*

Gels were cast between glass plates (de Wachter & Fiers, 1971). The plates were separated by Perspex side pieces 3 mm thick and along one edge was placed a "comb" of Perspex, which moulded the sample wells in the gel. The Perspex pieces were sealed to the glass plates with silicone grease and the plates clamped together with Bulldog clips. The assembly was stood with the comb along the lower edge. Agarose solution (Sigma electrophoresis grade agarose) was prepared by dissolving the appropriate weight in boiling electrophoresis buffer (E buffer of Loening, 1969). The solution was cooled to 60 to 70°C and poured into the assembly, where it was allowed to set for at least an hour. The assembly was then inverted, the comb removed and the wells filled with electrophoresis buffer. Samples made 5% with glycerol were loaded from a drawn-out capillary by inserting the tip below the surface and blowing gently. Electrophoresis buffer was layered carefully to fill the remaining space and a filter-paper wick inserted between the glass plates along the top edge. The lower end of the assembly was immersed in a tray of electrophoresis buffer containing the platinum anode, and the paper wick dipped into a similar cathode compartment. Electrophoresis was at 1·0 to 1·5 mA/cm width of gel for a period of about 18 h. Bromophenol blue marker travels about 3/4 the length of the gel under these conditions, but it should be noted that small DNA fragments move ahead of the bromophenol blue, especially in dilute gels. Cylindrical gels were cast in Perspex tubes 9 mm i.d. and either 12 or 24 cm long. These were run at 3 to 5 mA/tube in standard gel electrophoresis equipment.

Dr J. Spiers donated ribosomal DNA that had been purified on actinomycin/caesium chloride gradients from DNA made from the pooled blood of several animals, and also ^{3}H-labelled 18 S and 28 S RNAs prepared from cultured *Xenopus laevis* kidney cells. *Escherichia coli* DNA was prepared by Marmur's (1961) procedure from strain MRE600. ^{32}P-labelled *E. coli* RNA was prepared from cells grown in low phosphate medium with ^{32}Pi at a concentration of 50 μCi/ml and fractionated by electrophoresis on 10% acrylamide gels. ^{32}P-labelled rat DNA was a gift of M. S. Campo. DNA from human placenta was a gift of H. J. Cooke, DNA from rat liver was a gift of A. R. Mitchell, DNA from mouse and rabbit livers were gifts of M. White. Calf thymus DNA was purchased from Sigma Biochemicals. For digestion with restriction endonucleases, the DNAs were dissolved in water to a concentration of approximately 1 mg/ml. One-tenth volume of the appropriate buffer was added and sufficient enzyme to give a complete digestion overnight at 37°C. Enzyme activity was checked on phage λ DNA and digests of this DNA were also used as size markers in gel electrophoresis, using the values given by Thomas & Davis (1975).

(c) *Method of transfer*

This section describes the method finally adopted: preliminary experiments and controls are described in later sections.

After electrophoresis, the gel is immersed for 1 to 2 h in electrophoresis buffer containing ethidium bromide (0·5 μg/ml), and photographed in ultraviolet light (254 nm) with a red filter on the camera. A rule laid alongside the gel aids in matching the photograph of the fluorescence of the DNA to the final radioautograph of the hybrids. Strips to be used for transfer from flat gels are cut from the gel using a flamed blade. The strips should be 0·5 cm to 1 cm wide and normally extend from the origin to the anode end of the gel. The gels used in this laboratory are 3 mm thick, and the length from the origin to the anode end is 18 cm but the method can be adapted to gels with different dimensions and to cylindrical gels. Strips of gel are then transferred to measuring cylinders containing 1·5 M-NaCl, 0·5 M-NaOH for 15 min and this solution is then replaced by 3 M-NaCl, 0·5 M-Tris·HCl (pH 7) and the gel is left for a further 15 min. The depth of liquid in the cylinders should be greater than the length of the gel strips and the cylinders should be inverted from time to time. For cylindrical gels (9 mm diam.), the times required for denaturation and neutralization are 30 and 90 min. Each gel transfer requires:

One piece of thick filter paper 20 cm × 18 cm, soaked in 20 × SSC (SSC is 0·15 M-NaCl, 0·015 M-sodium citrate).

Two pieces of thick filter paper 2 cm × 18 cm soaked in 2 × SSC.

One strip of cellulose nitrate filter (e.g. Millipore 25 HAWP), 2·2 cm × 18 cm, soaked in 2 × SSC. These strips are immersed first by floating them on the surface of the solution; otherwise air is trapped in patches, which leads to uneven transfer.

Three pieces of glass or Perspex, 5 cm × 20 cm and the same thickness as the gel.

Four or five pieces of thick, dry filter paper, 10 cm × 18 cm.

Transfer of the denatured DNA fragments is carried out as follows.

The large filter paper soaked in 20 × SSC is laid on a glass or plastic surface, care being taken to avoid trapping air bubbles below the paper. 20 × SSC is poured on so that the surface is glistening wet. One of the glass or Perspex sheets is laid on top of the wet paper. The gel strip is taken from the neutralizing solution and laid parallel to the glass or Perspex sheet, 2 to 3 mm away from it. The second glass or Perspex sheet is laid 2 to 3 mm away from the other side of the gel (Fig. 1(a)). The cellulose nitrate strip is then laid on top of the gel with its edges resting on the sheets of Perspex or glass, so that it bridges the two air spaces (Fig. 1(b)). The two narrow pieces of filter paper, moistened with 2 × SSC are laid with their edges overlapping the cellulose nitrate strip by about 5 mm (Fig. 1(c)) and the dry filter paper is then placed on top of these (Fig. 1(d)).

For cylindrical gels, the arrangement is similar, but in this case, the Perspex that supports the Millipore filter may be in contact with the gel because an air space is retained over the top of the gel. Several cylindrical gels can be transferred at the same time using the apparatus shown in Fig. 2 and similar arrangements can be used for flat gels.

20 × SSC passes through the gel drawn by the dry filter paper and carries the DNA, which becomes trapped in the cellulose nitrate. The minimum time required for complete transfer has not been measured: it depends on the size of the fragments and probably also depends on the gel concentration. A period of 3 h is enough to transfer completely all HaeIII fragments of *E. coli* DNA from 2% agarose gels 3 mm thick. But even after 20 h, transfer of large EcoRI fragments of mouse DNA from 9 mm diam. cylindrical gels is not complete. DNA remaining in the gel can be seen by the fluorescence of the ethidium bromide, which is not completely removed during treatment of the gel. During the period of the transfer, it is necessary occasionally to add more 20 × SSC to the bottom sheet of filter paper. If the paper dries too much, the gel shrinks against the cellulose nitrate strip and liquid contact is broken. The paper may be flooded, but care must be taken that liquid does not fill the air spaces between the gel and the side-pieces and soak the paper, bypassing the gel. It may be found convenient to leave the cellulose nitrate in position overnight: if the supply of

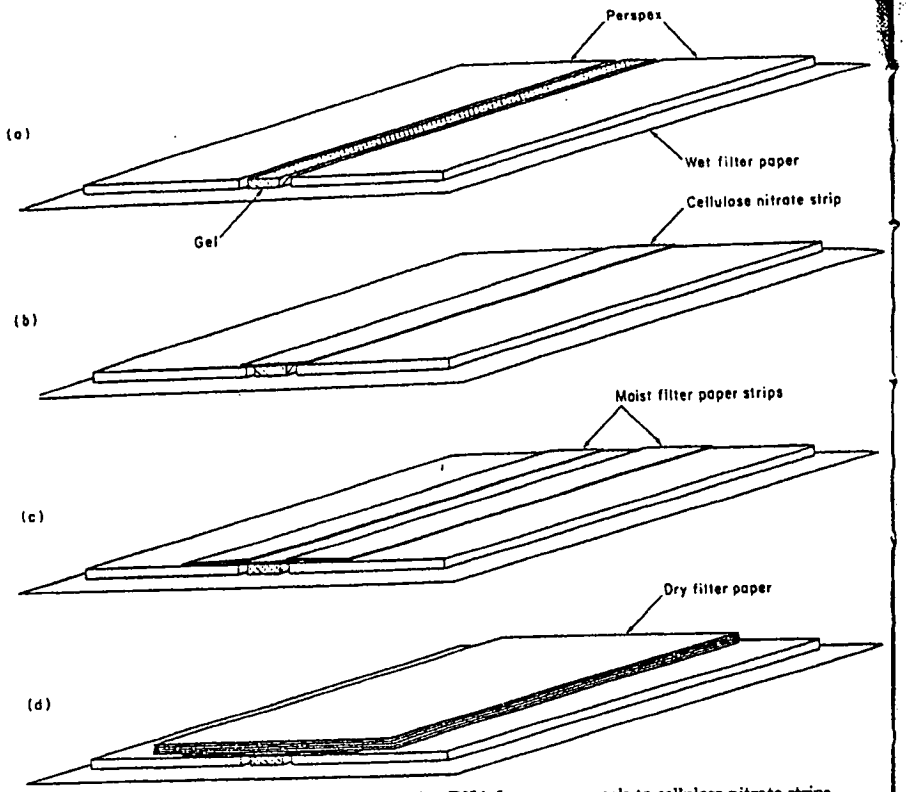

FIG. 1. Steps in the procedure for transferring DNA from agarose gels to cellulose nitrate strips.

20 × SSC has dried up it will be found that the gel has shrunk against the cellulose nitrate, but this does not impair the transfer. At the end of the transfer period the cellulose nitrate strip is lifted carefully so that the gel remains attached to its underside. It is turned over and the outline of the gel marked in pencil by a series of dots. The gel is peeled off the cellulose nitrate, the area of contact cut out with a flamed blade, and immersed in 2 × SSC for 10 to 20 min. The strip is then baked in a vacuum oven at 80°C for 2 h.

(d) *Hybridization*

Radioactive RNAs are usually available in small quantities only and it is important to keep the volume of the solution used for hybridization as small as possible so that the RNA has a reasonable concentration. Two procedures can be used for hybridizing the cellulose nitrate strips after transferring the restriction fragments.

The procedure that uses the smallest volume is carried out by moistening the strip in hybridization mixture and then immersing it in paraffin oil. A drop of RNA solution (0·3 ml for a strip 1 cm × 18 cm) is placed on a plastic sheet. One end of the

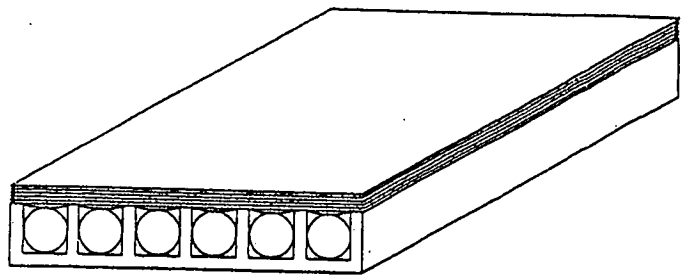

FIG. 2. Apparatus for transferring DNA from a number of cylindrical gels.

The apparatus is constructed of Perspex. The uprights which separate the gels and support the sheet of cellulose nitrate should be about 0·5 mm higher than the diameter of the gels, so that the cellulose nitrate sheet dips down to touch the gel. Thus an air gap is left between the cellulose nitrate sheet and the filter paper, above the line of contact between the gel and cellulose nitrate sheet. The apparatus is laid in a shallow tray containing 20 × SSC and the gels are then inserted into the troughs, care being taken to avoid trapping air bubbles beneath the gel. The cellulose nitrate sheet, wet with 2 × SSC, is laid over the gels and one piece of wet filter paper is laid over this. A stack of dry filter paper is then placed over the whole assembly. If necessary, a glass plate can be used to weigh down the filter papers. The depth of 20 × SSC in the tray should be enough to cover the lower part of the gels, but not so much that the air space between the Perspex and the cellulose nitrate becomes flooded.

cellulose nitrate strip is floated on the drop and when liquid is seen to soak through, the strip is drawn slowly over the surface of the drop. When it is completely wetted from one side, it is turned over and any remaining liquid is used to wet the other side. The strip is then immersed in paraffin oil saturated with the hybridization solution at the hybridization temperature. It should be borne in mind that baking the strip in 2 × SSC introduces salt, which must be taken into account when deciding on a solvent for the RNA if this method of hybridization is used. For example, if hybridization is to be carried out in 6 × SSC the RNA should be dissolved in 4 × SSC. Though this method can give good results (see Plate I) it often leads to high and uneven background. Kourilsky *et al.* (1974) found that this problem is removed if the hybridization is carried out in 2 × SSC, 40% formamide at 40°C. I have not tried this method, because this solvent removed DNA from the filters (see later section). It may well be the best method for hybridization to large fragments. I have found it convenient to carry out the hybridization in a vessel designed to hold the strip in a small volume of liquid.

The vessel (Fig. 3), which is easily made from Perspex, has internal dimensions of 0·8 mm deep by 2 cm high and about 1 cm longer than the strip to be hybridized. The vessel is filled with the solvent to be used for hybridization and the strip is fed in through the narrow opening in the top. The solvent is then drained off and the RNA solution introduced. Around 1 ml of solution is needed for a strip 1 cm × 18 cm. The wide sheets of cellulose nitrate used for transferring several gels (e.g. using the apparatus shown in Fig. 2) are too wide to be hybridized in this type of vessel. They can be hybridized in a small volume by wrapping them around a cylinder of Perspex, which is then inserted into a close-fitting tube. In this way, it is possible to hybridize a sheet 24 cm × 8 cm with about 4 ml of solution. If hybridization is carried out in a water-bath, it is not necessary to seal the top of the vessel provided the water-bath

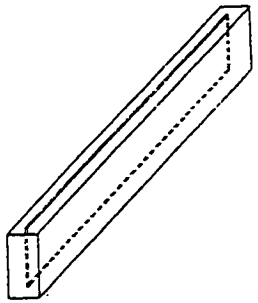

FIG. 3. Vessel used for hybridization of narrow strips.

itself is covered. The liquid in the vessel evaporates very slowly and can be replenished by small additions of water. A further advantage of this method of hybridization is that the RNA can be recovered and used again.

The period allowed for hybridization depends on the RNA concentration, its sequence complexity, its purity, and on the conditions of hybridization (see for example Bishop, 1972). After the appropriate period, strips are removed from the solution or paraffin oil, blotted between sheets of filter paper and washed, with stirring, for 20 to 30 min in a large volume of the hybridization solvent at the hybridization temperature. If the background is high, they may then be treated with a solution of RNAase A (20 μg/ml in 2 × SSC for 30 min at 20°C). After a final rinse in 2 × SSC they are dried in air.

So far the method has been tested with ^{32}P, ^{3}H, ^{35}S and ^{125}I-labelled RNAs. [^{32}P]RNAs have been detected by radioautography. For this the cellulose nitrate strips are laid on X-ray film and flattened against it with light pressure. ^{3}H, ^{125}I, ^{35}S and ^{14}C may be detected by fluorography. The cellulose nitrate strip is dipped through a solution of PPO in toluene (20%, w/v) dried in air, laid against X-ray film (Kodak RP-Royal Xomat) and kept at −70°C.

(e) *Completeness of transfer and retention of DNA*

Preliminary experiments showed that loading of DNA on to cellulose nitrate filters in 6 × SSC, conditions widely used in hybridization work, did not give complete retention of small fragments and a systematic study was made of the effect of salt concentration on retention. ^{3}H-labelled *X. laevis* DNA was sonicated to a single-strand molecular weight of 10^4 and denatured by boiling in 0·1 × SSC. Samples were made up to various salt concentrations and 0·1-ml portions of these solutions were pipetted on to cellulose nitrate filters, previously moistened with 2 × SSC, which were resting on glass-fibre filters. The solution that passed through the cellulose nitrate filter was thus collected in the glass-fibre filter. Both filters were then immersed in 5% trichloroacetic acid for 10 min, dried for 30 min in a vacuum oven at 80°C, and counted. It can be seen (Fig. 4) that the fraction of DNA retained by the cellulose nitrate increases with the salt concentration, and at concentrations above 10 × SSC the DNA is almost completely retained.

Losses of DNA at various stages of the transfer procedure were measured using ^{32}P-labelled *E. coli* DNA. The DNA was digested with EcoRI to give fragments in

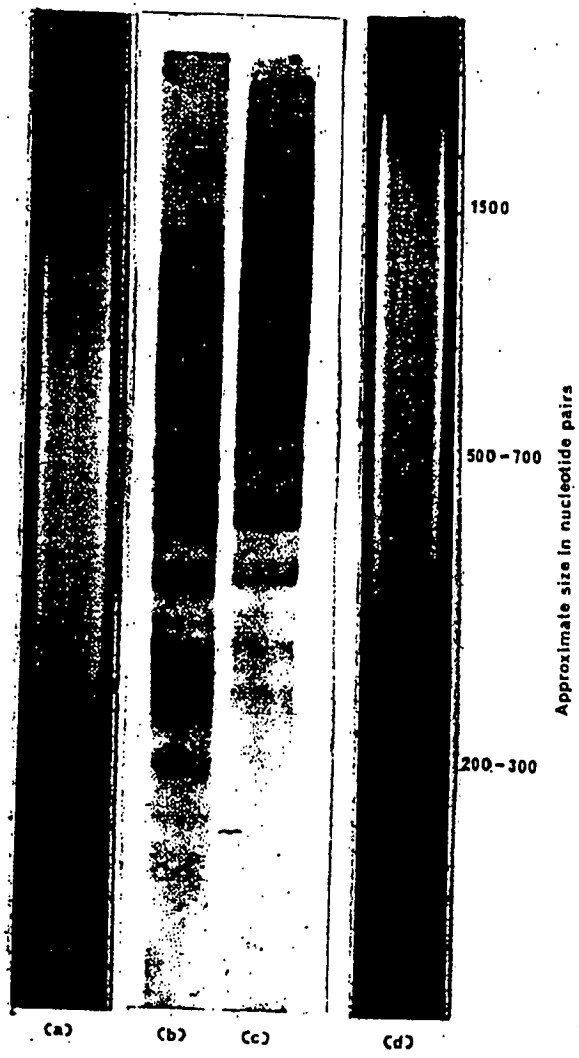

PLATE I. HaeIII digest of *E. coli* MRE600 DNA analyzed by electrophoresis on 2% agarose gel. DNA was then transferred to cellulose nitrate and hybridized with ^{32}P-labelled, high molecular weight RNA. (a) and (d) Photographs of ethidium bromide fluorescence. (b) and (c) Radioautographs of hybrids.

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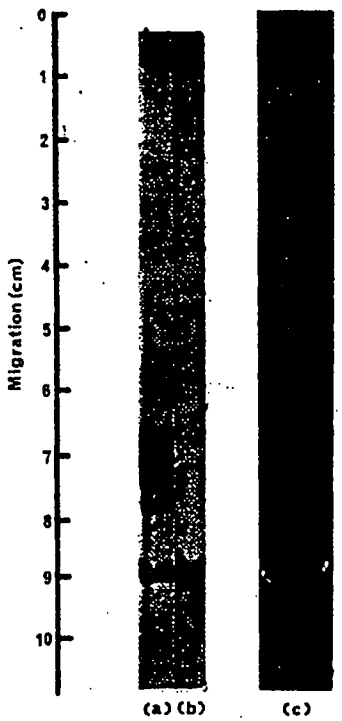

PLATE II. EcoRI digest of purified *X. laevis* ribosomal DNA analyzed by electrophoresis on 1% agarose gel. DNA was transferred to a cellulose nitrate strip, which was then cut longitudinally in two. The left-hand side was hybridized to 18 S RNA and the right-hand side to 28 S RNA (spec. act. of RNAs, $1{\cdot}5 \times 10^6$ c.p.m. per μg). Hybridization was done in $1 \times$ SSC at 65°C using the vessel shown in Fig. 3. A large excess of cold 28 S RNA was added to the labelled 18 S RNA to compete out any 28 S contamination. After hybridization, the strips were washed in $1 \times$ SSC at 65°C for 1·5 h, and dried. They were then dipped through a solution of PPO in toluene (20%, w/v) dried in air and placed against Kodak RP Royal X-ray film at −70°C for 2 months. Photograph of ethidium bromide fluorescence (c). Fluorograph of 18 S hybrids (a). Fluorograph of 28 S hybrids (b).

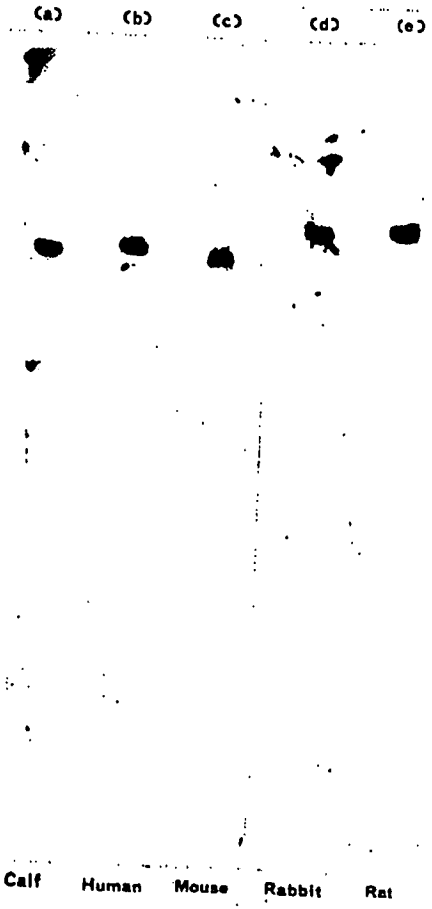

PLATE III. EcoRI digests of five mammalian DNAs, hybridized to 28 S RNA. Calf (a), human (b), mouse (c), rabbit (d) and rat (e) DNAs were digested to completion with EcoRI and separated by electrophoresis on 1% agarose gels (9mm × 12 cm, approx. 40 μg DNA per tube, 3 mA/tube for 16 h). The gels were pretreated as usual and the DNA fragments transferred to a single sheet of cellulose nitrate filter (12 cm × 8 cm) using the apparatus shown in Fig. 2. The top end of each gel was carefully aligned with one edge of the cellulose nitrate sheet. After 20 h, traces of DNA could still be seen, by ethidium bromide fluorescence, in the high molecular weight region of the gel. The filter was hybridized with 28 S RNA and radioautographed as described in the legend to Fig. 8.

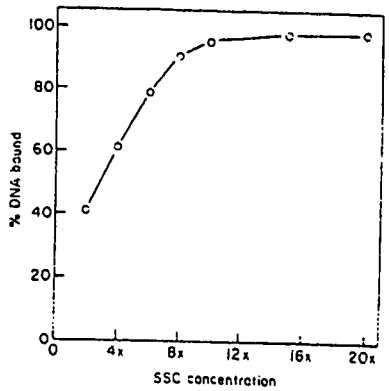

FIG. 4. Effect of salt concentration on efficiency of binding sonicated DNA to cellulose nitrate filters.

the large size range and with HaeIII to give small fragments. The fragments were then separated on a flat 1% agarose gel and transferred in the usual way. The solutions, the gel and the cellulose nitrate strip were counted. It can be seen (Table 1) that, whereas a small proportion of the DNA is leached out into the solutions during denaturation and neutralization, only traces remain in the gel after transfer.

TABLE 1

Losses of DNA at stages of the procedure

	EcoRI fragments	HaeIII fragments
	DNA lost (%)	
Denaturing solution	2·1	4·8
Neutralizing solution	1·3	4·4
Remaining in gel after transfer	0·21	0·31

Two samples of *E. coli* DNA (0·1 μg; spec. act. approx. 10^6 c.p.m. per μg) were digested with EcoRI and HaeIII. The fragments were separated by electrophoresis on 1% gels in 1-cm wide slots, and then transferred to cellulose nitrate strips as described in Materials and Methods. The transfer was left overnight. The radioactivity leached out of the gel by the denaturing and neutralizing solutions, that remaining in the gel, and that which had been trapped on the cellulose nitrate filter were measured in a liquid scintillation counter (Cerenkov radiation).

(f) *Effect of DNA size on yield of hybrid*

Melli & Bishop (1970) have shown that hybridization by the filter method gives low yields with low molecular weight DNA. Their results were obtained using a single set of hybridization conditions and it seemed possible that losses might be reduced by using high salt concentrations. The effect of salt concentration on loss of

DNA from the filters was examined by loading filters with radioactive *X. laevis* DNA, single-strand molecular weight about 10^4, and incubating them in various salt solutions at different temperatures. Increasing the salt concentration does improve the retention of the DNA at any given temperature (Table 2) but the gain does not appear to be useful, because with increasing salt concentration it is necessary to use higher temperatures for hybridization, and this cancels the advantage of the high salt concentration. For example, the loss in 2 × SSC at 65°C is the same as that in 6 × SSC at 80°C and these are both typical hybridization conditions. Further experiments showed that it is disadvantageous to perform hybridization at high salt concentrations, below the optimum temperature. The optimum temperature for rate of hybridization of *X. laevis* 28 S RNA is around 80°C in 6 × SSC but the rate at 70°C is still appreciable (Fig. 5). Below 70°C the rate falls rapidly. 28 S RNA was hybridized

TABLE 2

Effects of temperature and solvent on retention of sonicated DNA on cellulose nitrate filters

Solvent	Temperature 50°C	65°C	80°C	90°C
	DNA retained (%)			
2 × SSC		77	62	48
6 × SSC		97	76	56
10 × SSC		95	83	73
20 × SSC		97	88	81
6 × SSC in 50% formamide	58	50		

^{3}H-labelled *X. laevis* DNA (spec. act. approx. 5×10^5 c.p.m. per μg) was dissolved in ice-cold 0·1 × SSC and sonicated in six 15-s bursts. Between each treatment the solution was cooled in ice for 1 min. The solution was boiled for 5 min, made to 20 × SSC and cooled. Samples of this solution were pipetted on to 13-mm circles of cellulose nitrate, which were then washed in 2 × SSC at room temperature. Approximately 650 c.p.m. were loaded on each filter, and there was no loss caused by washing in 2 × SSC. The filters were dried, baked at 80°C for 2 h in a vacuum oven and immersed in 10 ml of the solvent equilibrated at the temperature used for incubation. After 90 min, the filters were removed, washed in 2 × SSC at room temperature, dried under vacuum and counted in a liquid scintillation counter.

to high molecular weight and sonicated DNA in 6 × SSC at 70 and 80°C (Fig. 6). As expected, the rate of hybridization at 70°C was lower than the rate at 80°C, but against expectation, both the rate and the final extent of hybridization were lower at the lower temperature, for the sonicated but not for the high molecular weight DNA. This result was unexpected because Melli & Bishop did not find an effect of DNA size on the rate of hybridization. They suggested that the decrease in yield for low molecular weight DNA is due to a loss of hybrid from the filter and it would be expected that such losses would increase with temperature. The lower yield for low molecular weight DNA at low temperature remains unexplained, but shows that there is no advantage to be gained in using high salt concentrations and low temperatures to retain small fragments of DNA during hybridization reactions. The advantage of using 6 × SSC at optimum temperature is that the rate is greatly increased over the rate with, say, 2 × SSC. A disadvantage is that the background of RNA that sticks to filters that have no DNA, increases with increasing salt concentration.

(g) *Methods of detecting and measuring hybrids: advantages of film detection*

Radioactive RNA may be detected and measured either by radioautography (or fluorography for weak β-emitters) or by cutting the strip into pieces, which can be counted in a scintillation counter. Film detection methods have the advantages over

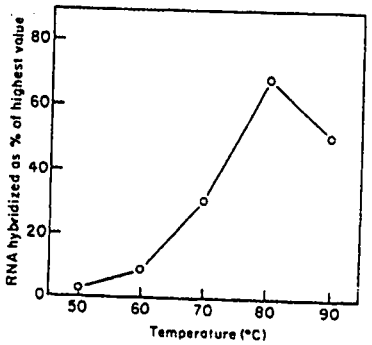

FIG. 5. Temperature dependence of hybridization of 28 S rRNA to *X. laevis* DNA.

X. laevis DNA was loaded on cellulose nitrate filters (17 μg DNA/13-mm diameter disc), which were pretreated as usual for hybridization. ^{3}H-labelled 28 S RNA from *X. laevis* kidney cells (spec. act. $1{\cdot}5 \times 10^6$ c.p.m./μg) was dissolved in 6 × SSC (0·28 μg/ml) and warmed to the temperature used for hybridization. Two filters loaded with DNA and 2 blank filters were introduced into the solutions and left for 30 min. They were washed in 2 l of 2 × SSC at room temperature, treated with 200 ml of RNAase A (20 μg/ml in 2 × SSC) at room temperature for 20 min, washed in 200 ml of 2 × SSC for 10 min, dried under vacuum and counted. Hybridization is expressed as a percentage of that obtained after 5 h at 80°C.

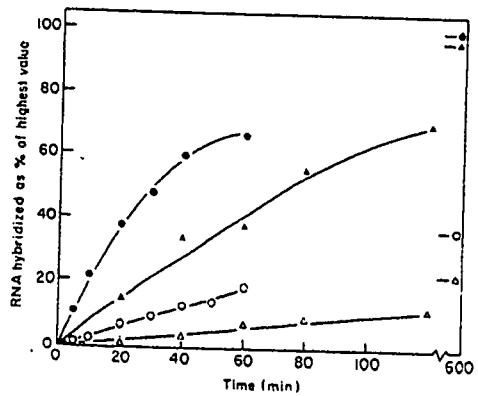

FIG. 6. Time course of hybridization of 28 S RNA to sonicated and high molecular weight DNA at 70 and 80°C.

Filters were loaded as described in the legend to Fig. 5. Two sets were loaded: one with high molecular weight DNA and one with DNA sonicated as described in the legend to Table 2. Hybridization and subsequent treatment of the filters was carried out as described in the legend to Fig. 6 and filters removed at the times indicated. 6 × SSC at 80°C, high molecular weight DNA (●); 6 × SSC at 70°C, high molecular weight DNA (▲); 6 × SSC, 80°C sonicated DNA (○); 6 × SSC at 70°C, sonicated DNA (△).

counting that they are more sensitive, give higher resolution, and can reveal artifacts not seen by counting.

The high sensitivity is illustrated by the analysis of *E. coli* rDNA (Plate I(b)). None of the bands that is clearly visible in the radioautograph contained more than 10 c.p.m. The strip of cellulose nitrate was cut into 150, 1-mm pieces and the pieces counted in a liquid scintillation counter. None of the pieces gave counts more than twice background and none of the features visible in the radioautograph was discernible from the counts. Around 100 c.p.m. of ^{32}P in a single band 1 cm wide can be detected with an overnight exposure. The radioautograph shown in Plate I was exposed for 1 week. Fluorography of ^{3}H is not so sensitive; about 3000 d.p.m. in a 1-cm band are needed to give a visible exposure overnight. The fluorograph shown in Plate II was exposed for 2 months.

The greater resolution of film detection is illustrated by a comparison of Plate II with Figure 7(c). Plate II is a fluorograph of the strip and Figure 7(c) shows the pattern of counts obtained by cutting the strip into 1-mm pieces. Many of the bands seen in the fluorograph are not discernible in the pattern of counts (compare also the tracing of the fluorograph (Fig. 7(b)) with (c)).

For ionizing radiation, blackening of the X-ray film is proportional to the amount of incident radiation, up to the limit where a high proportion of silver grains are exposed. The relative amount of radioactivity in bands can therefore be compared by tracing radioautographs in a densitometer and comparing peak areas. However, like all other photosensitivie materials, X-ray films suffer from "reciprocity failure" at low intensities of illumination by non-ionizing radiation and it is likely that bands which contain only a few counts of ^{3}H will not be detected by fluorography even after long exposures. I have not determined the lower limit of detection. Bonner & Laskey (1974) found that 500 d.p.m. of ^{3}H in a band 1 cm × 1 mm could be detected in one week and in my own experience, less than 20 d.p.m. can be detected with longer exposure. Reciprocity failure could affect quantitation of fluorographs by densitometry but comparison of Figure 7(b) and (c) suggests that the response of the film is linear within the limits of this experiment. Clearly, quantitation of ^{32}P by densitometry can be accurate and more sensitive than counting, but film response to ^{3}H may not be linear for low amounts.

An additional advantage of film detection is that non-specific binding of RNA to the cellulose nitrate is more easily distinguished from bands of hybrid. Plate III illustrates this point. In this radioautograph, non-specific binding can be seen as dots and streaks with an appearance clearly different from that of a band. Had this strip been analysed by counting, non-specific binding would not have been distinguishable from the hybrids.

(h) *Analysis of ribosomal DNA in* X. laevis

A total of 0·6 μg of purified *X. laevis* rDNA was digested with EcoRI and the fragments separated by electrophoresis in 1% agarose gels (Plate II(c)). The pattern of fragments is similar to that described by Wellauer *et al.* (1974). They compared the secondary structures of the denatured DNA fragments with those of the ribosomal RNAs and showed that the fastest running fragment (M_r approx. 3×10^6) contained most of the DNA coding for 28 S RNA, all of the transcribed spacer, and a small portion of the DNA coding for 18 S RNA. The larger fragments (M_r 4 to 6×10^6) contained most of the DNA coding for 18 S RNA, all of the non-transcribed

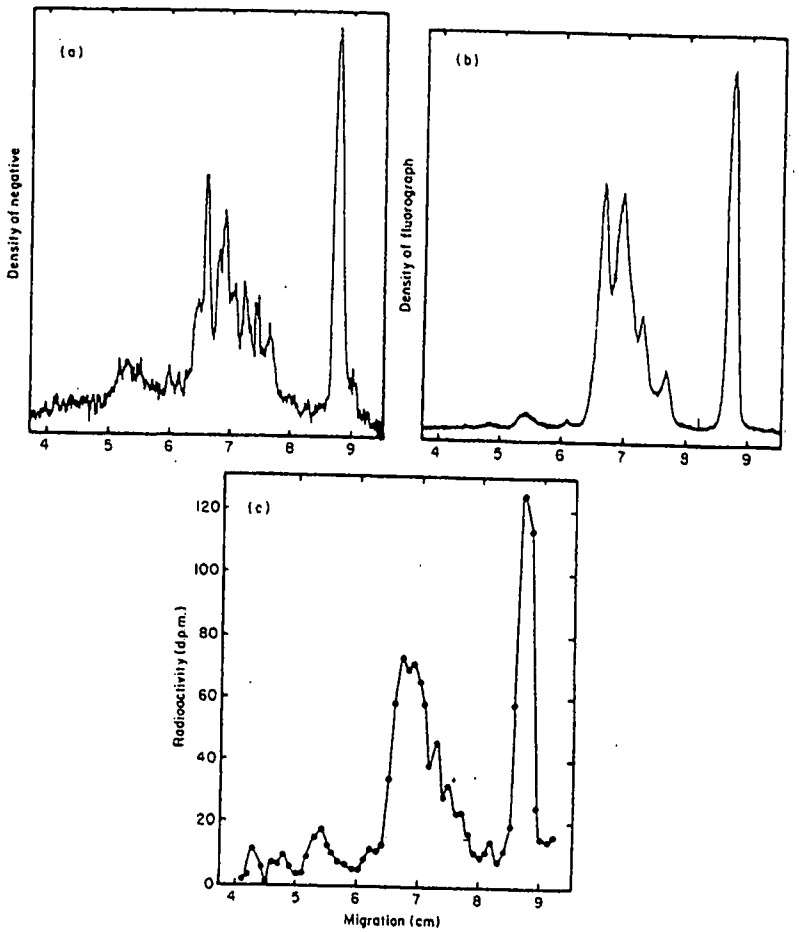

FIG. 7. (a) Microdensitometer tracing of the negative of Plate II(c). (b) Microdensitometer tracing of Plate II(a). (c) Distribution of counts in the Millipore strip which on fluorography gave Plate II(a). The strip was cut into 1 mm pieces, which were counted in a liquid scintillation counter at an efficiency of 40%.

spacer, and a small portion of the DNA coding for 28 S RNA. Different lengths of non-transcribed spacer DNA accounted for the variation in size of the longer fragments. The digest shown in Plate II(c) was transferred to cellulose nitrate as described previously. The strip was cut longitudinally into 2 parts and 1 part was hybridized with 18 S RNA and the other with 28 S RNA. Hybrids were detected by fluorography of the ^{3}H-labelled RNA (Plate II(a) and (b)). Comparison of Plate II(a) and (c)

shows that the resolution of the fine bands containing the 18 S coding sequence is not as high in the fluorograph as it is in the photograph of the gel. Whereas 9 bands can be distinguished in the photograph, only 7 can be distinguished with confidence in the fluorograph. From this analysis it is possible to locate the EcoRI site within the DNA coding for 18 S RNA. As Wellauer *et al.* (1974) showed, 1 of the 2 breaks in the rDNA occurs towards one end of the 18 S region and the other is close to the distal end of the 28 S region. The 3×10^6 mol. wt fragment accounts for virtually all of the hybridization to 28 S RNA and for about 30% of the hybridization to the 18 S RNA (27% measured from the tracing of the fluorograph (Fig. 7(b)) and 31% from the counts). Only traces of 28 S RNA hybridize to the heterogeneous collection of fragments with molecular weights between 4 and 6×10^6, whereas about 70% of the 18 S hybridization is accounted for in these fragments. Thus the break in the 28 S region of the DNA is very close to the end of the coding sequence and the break in the 18 S region is about one-third of the way into the coding sequence.

(i) *Analysis of mouse and rabbit ribosomal DNAs: evidence for long, non-transcribed spacer DNA*

An EcoRI digest of total mouse DNA was separated by electrophoresis on cylindrical 1% agarose gels and transferred to strips of cellulose nitrate paper. One strip was hybridized to 18 S RNA and another to 28 S RNA prepared from rat myoblasts labelled with ^{32}P. The 28 S hybrids showed a strong, sharp band at the position of about $5{\cdot}2\times10^6$ daltons and a very faint, broad band in the region around 14×10^6 daltons (Fig. 8(b)). The 18 S hybrids showed corresponding bands but in this case the slower moving, broad band was relatively more intense (Fig. 8(a)). From this information, a partial structure can be derived for the ribosomal DNA in mouse. Assuming that the ribosomal genes are tandemly linked, it is clear that EcoRI makes at least 2 breaks in the sequence; one in the 18 S and one in the 28 S region. Transcription of ribosomal genes in mammals produces a precursor RNA corresponding to a DNA mol. wt of about 6×10^6, and it follows that the EcoRI fragment of about $5{\cdot}2\times10^6$, which contains both 28 S and 18 S sequences, must also encompass much of the transcribed spacer. The heterogeneous fragments with a mol. wt of 14×10^6 must contain a long stretch of non-transcribed spacer, and may contain some of the transcribed spacer too.

A similar analysis was carried out with rabbit DNA and gave similar results, although the size of the fragments was different from the corresponding fragments from mouse DNA. The band containing most of the 28 S sequence was larger (M_r approx. 6×10^6), whereas that containing most of the 18 S sequence was smaller (M_r approx. 12×10^6) and more homogeneous than the corresponding fragment in the mouse. The structures of mouse and rabbit ribosomal DNAs are thus rather similar to that of *X. laevis* but with longer spacer regions. The overall length of the unit in mouse is at least twice as long as that in *X. laevis*.

(j) *EcoRI sites in the rDNA of five mammals*

The analyses described above, taken with those of Wellauer *et al.* (1974) suggest that the two EcoRI sites in the ribosomal genes have been conserved since the amphibians and mammals diverged. In this case it would be expected that all

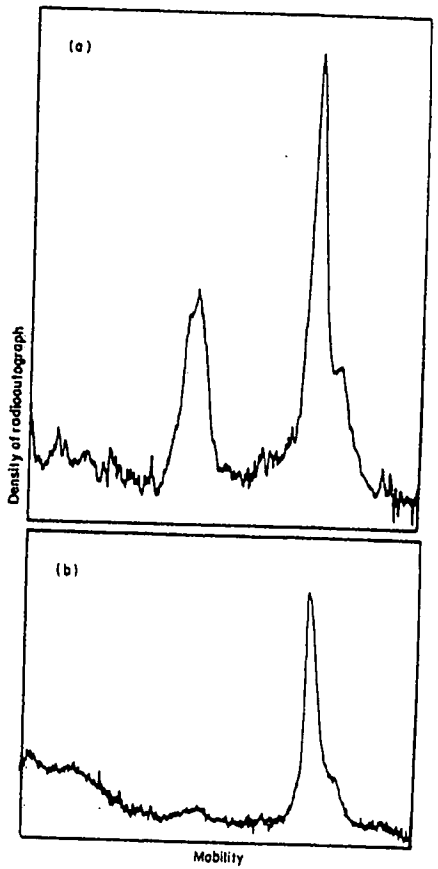

FIG. 8. EcoRI digest of mouse DNA hybridized to 18 S and 28 S RNA.

Total mouse DNA was digested to completion with EcoRI. The digest was separated by electrophoresis on 1% cylindrical agarose gels (9 mm × 24 cm, 5 mA/tube for 20 h, 40 μg of DNA/gel). The gels were stained, photographed, and the DNA transferred to cellulose nitrate as described in Materials and Methods. One gel was hybridized to ^{32}P-labelled 18 S RNA and another to 28 S RNA. The RNA concentration was 0·1 μg/ml in 6×SSC and hybridization was carried out at 80°C for 4 h. The filters were then washed in 2×SSC (4 l) at 60°C for 30 min, dried and radioautographed using Kodak Blue Brand X-ray film.

(a) Densitometer tracing of the 18 S hybrids. (b) Densitometer tracing of the 28 S hybrids.

mammalian rDNAs would have equivalent EcoRI sites. Total DNAs from calf thymus, human placenta, and from livers of mouse, rabbit and rat were digested with EcoRI and the fragments separated by electrophoresis on cylindrical gels. The fragments were then transferred to a single sheet of cellulose nitrate filter and hybridized with ^{32}P-labelled rat 28 S RNA. All 5 DNAs showed a strong band in the radioautograph

of the sheet. Each band was in the mol. wt region of 5 to 6×10^6, but there were small differences in their mobilities (Table 3). This result suggests that the two EcoRI sites have indeed been conserved in the rDNA of the mammals. The different fragment size

TABLE 3

Size of EcoRI fragments that hybridize to ribosomal RNAs

Species	Size of RI fragment bearing 28 S sequences ($\times 10^{-6}$)	Size of fragments bearing 18 S sequences ($\times 10^{-6}$)
Calf	5·7	
Human	5·7	
Mouse	5·2	5·2 and approx. 14
Rabbit	6·0	6·0 and approx. 12
Rat	6·0	
X. laevis	3·0	3·0 and 4 to 6

Sizes were estimated from mobilities in 1% agarose gels by comparison with EcoRI fragments of λ-phage DNA. The sizes of the large fragments from mouse and rabbit DNAs hybridizing to 18 S RNA are approximate estimates because there was only one marker in this region of the gel and in this region large differences in size result in small mobility differences.

can readily be accounted for by differences in the size of the transcribed spacer between 28 S and 18 S regions. Different sizes for the ribosomal RNA precursor have been reported for HeLa cells and mouse L-cells (Grierson *et al.*, 1970).

3. Conclusion

The method described here provides a simple way of detecting DNA fragments that are complementary to RNAs, after the DNA frqgments have been separated by gel electrophoresis. Transfer of the DNA from the gel to the cellulose nitrate filter is almost complete for a wide range of fragment sizes. However, large fragments ($M_r > 10^7$) diffuse rather slowly and small fragments hybridize inefficiently. These factors should be taken into account when the method is used for quantitative work.

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2

Molecular Cloning

A LABORATORY MANUAL

SECOND EDITION

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UNIVERSITY OF TEXAS SOUTHWESTERN MEDICAL CENTER

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GENETICS INSTITUTE

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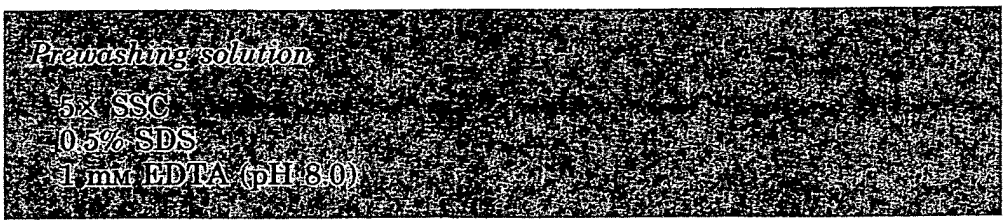
Hybridization to Nitrocellulose Filters Containing Replicas of Bacterial Colonies

The following protocol is designed for 30 circular nitrocellulose filters, 82 mm in diameter. Appropriate adjustments to the volumes should be made when carrying out hybridization reactions with different numbers or sizes of filters.

1. Float the baked filters on the surface of a tray of 2× SSC until they have become thoroughly wetted from beneath. Submerge the filters for 5 minutes.

2. Transfer the filters to a glass crystallizing dish containing at least 200 ml of prewashing solution. Stack the filters on top of one another in the solution. Cover the dish with Saran Wrap and transfer it to a rotating platform in an incubator. In this and all subsequent steps, the filters should be slowly agitated to prevent them from sticking to one another. Incubate the filters for 30 minutes at 50°C.

   Important: Do not allow the filters to dry at any stage during the prewashing, prehybridization, or hybridization steps.



3. Gently scrape the bacterial debris from the surfaces of the filters using Kimwipes soaked in prewashing solution. This reduces background hybridization without affecting the intensity or sharpness of positive signals.

4. Transfer the filters to 150 ml of prehybridization solution in a glass crystallizing dish. Incubate the filters for 1–2 hours at the appropriate temperature (i.e., 68°C when hybridization is to be carried out in aqueous solution; 42°C when hybridization is to be carried out in 50% formamide).

   Some workers prefer to incubate filters in heat-sealable plastic bags (Sears Seal-A-Meal or equivalent) (see, e.g., Chapter 9, page 9.53). This method avoids problems with evaporation and, because the sealed bags can be submerged in a water bath, ensures that the temperatures during hybridization and washing are correct. The bags must be opened and resealed when changing buffers. To avoid radioactive contamination of the water bath, the resealed bag containing radioactivity should be sealed inside a second, noncontaminated bag.

   Often, small bubbles of air form on the surface of the filter as the temperature of the prehybridization solution increases. It is important that these bubbles be removed by occasionally agitating the fluid in the bag; otherwise, the components of the prehybridization solution will not be able to coat the filter evenly. This problem can be minimized by heating the prehybridization solution to the appropriate temperature before adding it to the bag.

   The filters should be completely covered by the prehybridization solution. During prehybridization, sites on the nitrocellulose filter that nonspecifically bind single- or double-stranded DNA become blocked by proteins in the BLOTTO.

When ^{32}P-labeled cDNA or RNA is used as a probe, poly(A) at a concentration of 1 μg/ml should be included in the prehybridization and hybridization solutions to prevent the probe from binding to T-rich sequences that are found fairly commonly in eukaryotic DNA.

Whether or not to use a prehybridization solution containing formamide is largely a matter of personal preference. Both versions of these solutions give excellent results and neither has clear-cut advantages over the other. However, hybridization in 50% formamide at 42°C is less harsh on nitrocellulose filters than is hybridization at 68°C in aqueous solution. Offsetting this advantage is the two- to threefold slower rate of hybridization in solutions containing formamide.

To maximize the rate of annealing of the probe with its target, hybridizations are usually carried out in solutions of high ionic strength (6× SSC or 6× SSPE) at a temperature that is 20–25°C below T_m (see Chapter 9, pages 9.50–9.51). Both solutions work equally well when hybridization is carried out in aqueous solvents. However, when formamide is included in the hybridization buffer, 6× SSPE is preferred because of its greater buffering power.

Prehybridization solution

Either
50% formamide
6× SSC (or 6× SSPE)
0.05× BLOTTO

or
6× SSC (or 6× SSPE)
0.05× BLOTTO

Formamide: Many batches of reagent-grade formamide are sufficiently pure to be used without further treatment. However, if any yellow color is present, the formamide should be deionized by stirring on a magnetic stirrer with Dowex XG8 mixed-bed resin for 1 hour and filtering twice through Whatman No. 1 paper. Deionized formamide should be stored in small aliquots under nitrogen at −70°C.

1× BLOTTO: Bovine Lacto Transfer Technique Optimizer (Johnson et al. 1984) is 5% nonfat dried milk dissolved in water containing 0.02% sodium azide. It should be stored at 4°C. 1× BLOTTO is as effective a blocking agent as Denhardt's reagent, but much less expensive. BLOTTO should not be used in combination with high concentrations of SDS, which will cause the milk proteins to precipitate. If background hybridization is a problem, NP-40 may be added to the hybridization solution to a final concentration of 1%. BLOTTO should not be used as a blocking agent when radiolabeled RNA is used as the hybridization probe, because of the possibility that the dried milk may contain significant amounts of RNAase activity.

Caution: Sodium azide is poisonous. It should be handled with great care, wearing gloves, and solutions containing it should be clearly marked.

5. Denature ^{32}P-labeled double-stranded DNA probe by heating for 5 minutes to 100°C. Chill the probe rapidly in ice water. Single-stranded probe need not be denatured. Add the probe to the prehybridization solution covering the filters. Incubate at the appropriate temperature until $1-3 \times C_0t_{1/2}$ is achieved (see Chapter 9, page 9.48). During the hybridization, the containers holding the filters should be tightly closed to prevent the loss of fluid by evaporation.

   Alternatively, the probe may be denatured by adding 0.1 volume of 3 N NaOH. After 5 minutes at room temperature, transfer the probe to ice water and add 0.05 volume of 1 M Tris · Cl (pH 7.2) and 0.1 volume of 3 N HCl. Store the probe in ice water until it is needed.

   Between 2×10^5 and 1×10^6 cpm of ^{32}P-labeled probe (sp. act. $\geq 5 \times 10^7$ cpm/μg) should be used per milliliter of prehybridization solution. Using more probe will cause the background of nonspecific hybridization to increase; using less will reduce the rate of hybridization.

6. When the hybridization is completed, remove the hybridization solution and immediately immerse the filters in a large volume (300–500 ml) of 2× SSC and 0.1% SDS at room temperature. Agitate the filters gently, and turn them over at least once during washing. After 5 minutes, transfer the filters to a fresh batch of wash solution and continue to agitate them gently. Repeat the washing procedure twice more. At no stage during the washing procedure should the filters be allowed to dry.

   Hybridization mixtures containing radiolabeled single-stranded probes may be stored at 4°C for several days and reused without further treatment. However, hybridization mixtures containing complementary strands of DNA should be discarded since there is no satisfactory way to denature the double-stranded DNA that forms during the first round of hybridization.

7. Wash the filters twice for 1–1.5 hours in 300–500 ml of a solution of 1× SSC and 0.1% SDS at 68°C. At this point, the background is usually low enough to put the filters on film. If the background is still high or if the experiment demands washing at high stringencies, immerse the filters for 60 minutes in 300–500 ml of a solution of 0.2× SSC and 0.1% SDS at 68°C.

8. Dry the filters in the air at room temperature on paper towels. Arrange the filters (numbered side up) on a sheet of Saran Wrap. Apply adhesive dot labels marked with radioactive ink to several asymmetric locations on the Saran Wrap. These markers serve to align the autoradiograph with the filters. Cover the labels with Scotch Tape. This prevents contamination of the film holder or intensifying screen with the radioactive ink.

   Radioactive ink is made by mixing a small amount of ^{32}P with waterproof black drawing ink. We find it convenient to make the ink in three grades: very hot (>2000 cps on a hand-held minimonitor), hot (>500 cps on a hand-held minimonitor), and cool (>50 cps on a hand-held minimonitor). Use a fiber-tip pen to apply ink of the desired hotness to the adhesive labels. Attach radioactive-warning tape to the pen, and store it in an appropriate place.

1

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TABLE 9.1 Blocking Agents Used to Suppress Background in Hybridization Experiments

Agent	Recommended uses
Denhardt's reagent	northern hybridizations hybridizations using RNA probes single-copy Southern hybridizations hybridizations involving DNA immobilized on nylon membranes

Denhardt's reagent (Denhardt 1966) is usually made up as a 50× stock solution, which is filtered and stored at −20°C. The stock solution is diluted tenfold into prehybridization buffer (usually 6× SSC or 6× SSPE containing 0.5% SDS and 100 μg/ml denatured, fragmented salmon sperm DNA). 50× Denhardt's reagent contains 5 g of Ficoll (Type 400, Pharmacia), 5 g of polyvinylpyrrolidone, 5 g of bovine serum albumin (Fraction V; Sigma), and H_2O to 500 ml.

BLOTTO	Grunstein/Hogness hybridization Benton/Davis hybridization all Southern hybridizations other than single-copy dot blots

1× BLOTTO (Bovine Lacto Transfer Technique Optimizer; Johnson et al. 1984) is 5% nonfat dried milk dissolved in water containing 0.02% sodium azide. It should be stored at 4°C and diluted 25-fold into prehybridization buffer before use. BLOTTO should not be used in combination with high concentrations of SDS, which will cause the milk proteins to precipitate. If background hybridization is a problem, NP-40 may be added to the hybridization solution to a final concentration of 1%. BLOTTO should not be used as a blocking agent in northern hybridizations because of the possibility that it might contain unacceptably high levels of RNAase.

Caution: Sodium azide is poisonous. It should be handled with great care, wearing gloves, and solutions containing it should be clearly marked.

Heparin	Southern hybridization in situ hybridization

Heparin (Sigma H-7005 porcine grade II or equivalent) is dissolved at a concentration of 50 mg/ml in 4× SSPE or 4× SSC and stored at 4°C. It is used as a blocking agent at a concentration of 500 μg/ml in hybridization solutions containing dextran sulfate; in hybridization solutions that do not contain dextran sulfate, heparin is used at a concentration of 50 μg/ml (Singh and Jones, 1984).

Denatured, fragmented salmon sperm DNA	Southern and northern hybridizations

Salmon sperm DNA (Sigma type III sodium salt) is dissolved in water at a concentration of 10 mg/ml. If necessary, the solution is stirred on a magnetic stirrer for 2–4 hours at room temperature to help the DNA to dissolve. The concentration of NaCl is adjusted to 0.1 M, and the solution is extracted once with phenol and once with phenol:chloroform. The aqueous phase is recovered, and the DNA is sheared by passing it 12 times rapidly through a 17-gauge hypodermic needle. The DNA is precipitated by adding 2 volumes of ice-cold ethanol. It is then recovered by centrifugation and redissolved at a concentration of 10 mg/ml in water. The OD_{260} of the solution is determined and the exact concentration of the DNA is calculated. The solution is then boiled for 10 minutes and stored at −20°C in small aliquots. Just before use, the solution is heated for 5 minutes in a boiling-water bath and then chilled quickly in ice water. Denatured, fragmented salmon sperm DNA should be used at a concentration of 100 μg/ml in prehybridization solutions.

Hybridization of Radiolabeled Probes to Nucleic Acids Immobilized on Nitrocellulose Filters or Nylon Membranes

Although the method given below deals with RNA or DNA immobilized on nitrocellulose filters, only slight modifications are required to adapt the procedure to nylon membranes. These modifications are noted at the appropriate places in the text.

1. Prepare the prehybridization solution appropriate for the task at hand. Approximately 0.2 ml of prehybridization solution will be required for each square centimeter of nitrocellulose filter or nylon membrane.

   The prehybridization solution should be filtered through a 0.45-micron disposable cellulose acetate filter (Schleicher and Schuell Uniflow syringe filter No. 57240 or equivalent).

Prehybridization solutions

For detection of low-abundance sequences:

Either

6× SSC (or 6× SSPE)
5× Denhardt's reagent
0.5% SDS
100 μg/ml denatured, fragmented salmon sperm DNA

or

6× SSC (or 6× SSPE)
5× Denhardt's reagent
0.5% SDS
100 μg/ml denatured, fragmented salmon sperm DNA
50% formamide

For preparation of Denhardt's reagent and denatured, fragmented salmon sperm DNA, see Table 9.1.

Formamide: Many batches of reagent-grade formamide are sufficiently pure to be used without further treatment. However, if any yellow color is present, the formamide should be deionized by stirring on a magnetic stirrer with Dowex XG8 mixed-bed resin for 1 hour and filtering twice through Whatman No. 1 paper. Deionized formamide should be stored in small aliquots under nitrogen at −70°C.

For detection of moderate- or high-abundance sequences:

Either

6× SSC (or 6× SSPE)
0.05× BLOTTO

or

6× SSC (or 6× SSPE)
0.05× BLOTTO
50% formamide

For preparation of BLOTTO, see Table 9.1.

When ^{32}P-labeled cDNA or RNA is used as a probe, poly(A)$^+$ RNA at a concentration of 1 μg/ml may be included in the prehybridization and hybridization solutions to prevent the probe from binding to T-rich sequences that are found fairly commonly in eukaryotic DNA.

2. Float the nitrocellulose filter or nylon membrane containing the target DNA on the surface of a tray of 6 × SSC (or 6 × SSPE) until it becomes thoroughly wetted from beneath. Submerge the filter for 2 minutes.

3. Slip the wet filter into a heat-sealable bag (e.g., Sears Seal-A-Meal or equivalent). Add 0.2 ml of prehybridization solution for each square centimeter of nitrocellulose filter or nylon membrane.

   Squeeze as much air as possible from the bag. Seal the open end of the bag with the heat sealer. Incubate the bag for 1–2 hours submerged at the appropriate temperature (68°C for aqueous solvents; 42°C for solvents containing 50% formamide).

   Often, small bubbles of air form on the surface of the filter as the temperature of the prehybridization solution increases. It is important that these bubbles be removed by occasionally agitating the fluid in the bag; otherwise, the components of the prehybridization solution will not be able to coat the filter evenly. This problem can be minimized by heating the prehybridization solution to the appropriate temperature before adding it to the bag.

4. If the radiolabeled probe is double-stranded, denature it by heating for 5 minutes at 100°C. Single-stranded probe need not be denatured. Chill the probe rapidly in ice water.

   Alternatively, the probe may be denatured by adding 0.1 volume of 3 N NaOH. After 5 minutes at room temperature, transfer the probe to ice water and add 0.05 volume of 1 M Tris · Cl (pH 7.2) and 0.1 volume of 3 N HCl. Store the probe in ice water until it is needed.

   For Southern hybridization of mammalian genomic DNA where each lane of the gel contains 10 μg of DNA, 10–20 ng/ml radiolabeled probe (sp. act. = 10^9 cpm/μg or greater) should be used. For Southern hybridization of fragments of cloned DNA where each band of the restriction digest contains 10 ng of DNA or more, much less probe is required. Typically, hybridization is carried out for 6–8 hours using 1–2 ng/ml radiolabeled probe (sp. act. = 10^9 cpm/μg or greater).

5. Working quickly, remove the bag containing the filter from the water bath. Open the bag by cutting off one corner with scissors. Add the denatured probe to the prehybridization solution, and then squeeze as much air as possible from the bag. Reseal the bag with the heat sealer so that as few bubbles as possible are trapped in the bag. To avoid radioactive contamination of the water bath, the resealed bag should be sealed inside a second, noncontaminated bag.

   When using nylon membranes, the prehybridization solution should be *completely* removed from the bag and immediately replaced with hybridization solution. The probe is then added and the bag is resealed.

Hybridization solution for nylon membranes

6× SSC (or 6× SSPE)
0.5% SDS
100 μg/ml denatured, fragmented salmon sperm DNA
50% formamide (if hybridization is to be carried out at 42°C)

6. Incubate the bag submerged in a water bath set at the appropriate temperature for the required period of hybridization.

7. Wearing gloves, remove the bag from the water bath and immediately cut off one corner. Pour out the hybridization solution into a container suitable for disposal, and then cut the bag along the length of three sides. Remove the filter and immediately submerge it in a tray containing several hundred milliliters of 2× SSC and 0.5% SDS at room temperature.

   Important: Do not allow the filter to dry out at any stage during the washing procedure.

8. After 5 minutes, transfer the filter to a fresh tray containing several hundred milliliters of 2× SSC and 0.1% SDS and incubate for 15 minutes at room temperature with occasional gentle agitation.

   If short oligonucleotides are used as probes, washing should be carried out only for brief periods (1–2 minutes) at the appropriate temperature. For a discussion of the stability of hybrids involving oligonucleotides, see Chapter 11.

9. Transfer the filter to a flat-bottom plastic box containing several hundred milliliters of fresh 0.1× SSC and 0.5% SDS. Incubate the filter for 30 minutes to 1 hour at 37°C with gentle agitation.

10. Replace the solution with fresh 0.1× SSC and 0.5% SDS, and transfer the box to a water bath set at 68°C for an equal period of time. Monitor the amount of radioactivity on the filter using a hand-held minimonitor. The parts of the filter that do not contain DNA should not emit a detectable signal. You should not expect to pick up a signal on the minimonitor from filters containing mammalian DNA that has been hybridized to single-copy probes.

11. Briefly wash the filter with 0.1× SSC at room temperature. Remove most of the liquid from the filter by placing it on a pad of paper towels.

12. Place the damp filter on a sheet of Saran Wrap. Apply adhesive dot labels marked with radioactive ink to several asymmetric locations on the Saran Wrap. These markers serve to align the autoradiograph with the filter. Cover the labels with Scotch Tape. This prevents contamination of the film holder or intensifying screen with the radioactive ink.

    Radioactive ink is made by mixing a small amount of ^{32}P with waterproof black drawing ink. We find it convenient to make the ink in three grades: very hot

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Table 16-3
Protein and Lipid Content of Organellar Membranes

Membrane	Approximate Protein/Lipid Ratio (wt/wt)	Approximate Cholesterol/ Other Lipids (Molar Ratio)
Golgi apparatus	0.7	0.08
Liver plasma membrane	1.0	0.40
Endoplasmic reticulum	1.0	0.06
Mitochondrial outer membrane	1.0	0.05
Mitochondrial inner membrane	3.0	0.03
Nuclear membrane	3.0	0.11
Lysosomal membrane	3.0	0.16

Adapted from M. H. Saier, Jr., and C. D. Stiles, *Molecular Dynamics in Biological Membranes*, Heidelberg Science Library, Vol. 22, Springer Verlag, New York, 1975.

a sucrose density gradient (column 4) does *not* correlate with size, but rather with its density, which is determined by its chemical composition (Figure 16-3). Nucleic acid ($\rho \sim 1.7$) is more dense than protein ($\rho \sim 1.25$), and protein is more dense than lipid ($\rho = 0.9$–1.1). These facts account for the relatively high density of nuclei and the low density of the Golgi apparatus, which has a relatively high lipid content. Since each organelle has a specific function, it also must possess a unique complement of enzymes. This prediction has been amply verified by the subcellular localization of numerous enzymes (Table 16-2, column 5), and these specific associations have greatly facilitated the assay and isolation of organelles from eukaryotic cells.

Once the subcellular organelles have been separated, their membranes can be isolated. For those organelles enclosed by a single membrane, treatment in hypotonic buffer (*osmotic shock*) followed by centrifugal separation of the membrane ghosts from the intraorganellar soluble proteins allows one to study membrane composition. Nuclei and mitochondria, however, possess two membranes, and these must be separated before their chemical and physical properties can be studied. In these cases, selective solubilization of the outer membrane can be obtained by treatment with appropriate detergents allowing purification of intact inner membranes. Procedures such as these have allowed detailed analyses of the lipid and protein contents of organellar membranes (Table 16-3) and have provided experimental systems in which to study the structures and functions of each different membrane system.

Constituents of Bacterial Cell Envelopes

In contrast to animal cells, most prokaryotic cells are surrounded by a rather complex and rigid *cell wall*, which allows bacteria to live in a hypotonic environment without bursting and confers upon these cells their characteristic shape (rod, sphere, or spiral). In 1884, Christian Gram dis

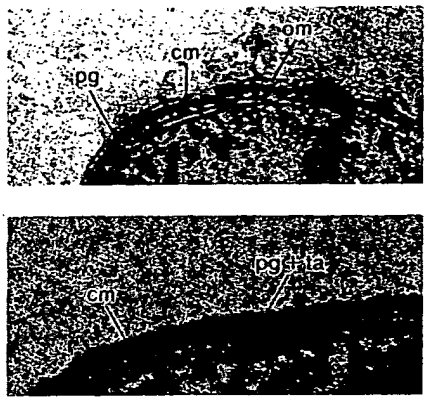

Figure 16-4
Electron micrographs of sections through the surface layers of a Gram-positive (*a*) and a Gram-negative (*b*) bacterium (cm = cytoplasmic membrane; om = outer membrane; Pg = peptidoglycan; ta = teichoic acid). Note the thick cell wall in (*a*) compared with the distinct inner and outer trilaminar membranes separated by a thin peptidoglycan layer in (*b*). (Courtesy J. Stolz; 150,000×.)

covered that bacteria could be divided into those which retained a crystal violet–iodine dye complex after washing with alcohol (*Gram positive*) and those which did not (*Gram negative*). Even today, the Gram stain reaction is a useful tool in classifying bacteria, and this difference in staining has been found to correlate with a fundamental difference in cell wall structure between Gram-positive and Gram-negative cells (Figure 16-4). Gram-positive cells are surrounded by a cytoplasmic membrane and a thick cell wall consisting of a sugar–amino acid heteropolymer, or *peptidoglycan* (Figure 16-5) and polyol phosphate polymers called *teichoic acids* (Figure 16-6*a*). Gram-negative bacteria have a much thinner cell wall consisting entirely of peptidoglycan and associated proteins, and this cell wall is surrounded by a second, outer membrane comprised of lipid, *lipopolysaccharide* (Figure 16-6*b*), and protein. The biosynthesis of the peptidoglycan and the outer membrane lipopolysaccharide are discussed in Chapter 12. The space between the inner and outer membranes, or *periplasmic space*, also contains proteins that have a variety of functions (see the following discussion).

In order to examine the compositions and functions of the various cell layers of Gram-negative bacteria, it is necessary to first separate these layers. This has been accomplished by treatment of the cells with *lysozyme* (which hydrolyzes peptidoglycan) and EDTA (which destabilizes the outer membrane) in isoosmotic sucrose solutions (Figure 16-7). *Periplasmic proteins* are released by this first step and can be separated by sedimenting the resulting *spheroplasts*, which have lost any nonspherical shape characteristic of the original cell because their peptidoglycan cell wall has been digested. Subsequent treatment of spheroplasts with high-frequency sound (*sonication*) ruptures both outer and inner membranes which quickly reseal into smaller spherical, closed *vesicles* (Figure 16-7). Because of their higher carbohydrate content, vesicles derived from the outer membrane have a higher density than those derived from the inner membrane and thus can be separated from them by isopycnic centrifugation in a sucrose density gradient. By these techniques, electron transport chains, ATP synthesizing enzymes, many transport proteins, and other enzymes have been

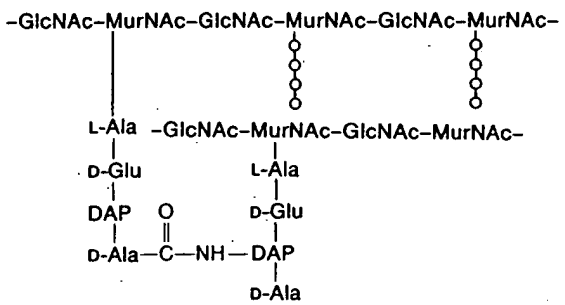

Figure 16-5
Two-dimensional representation of a bacterial peptidoglycan network. Chains of repeating *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) residues are linked by means of amide bonds between D-alanine and diaminopimelic acid (DAP) residues of tetrapeptides attached to the MurNAc units. Some tetrapeptides are not so linked (*vertical chains*).

3

Molecular Cloning

A LABORATORY MANUAL

SECOND EDITION

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Lysis of Cells

This is perhaps the most crucial step in immunoprecipitation. The aim is to find a method that will solubilize all of the target antigen in a form that is immunoreactive, undegraded, and, for some purposes, biologically active. In view of the wide range of physical and biological properties of mammalian proteins, it is not surprising that no single method of lysis is sufficient for every purpose. Among the variables that have been found to influence the efficiency of solubilization and subsequent immunoprecipitation of proteins are the ionic strength and pH of the lysis buffer; the concentration and type of detergent used; and the presence of divalent cations, cofactors, and stabilizing ligands.

Although there are exceptions, many soluble nuclear and cytoplasmic proteins can be solubilized by lysis buffers that contain the nonionic detergent Nonidet P-40 (NP-40) and either no salt at all or relatively high concentrations of salt (e.g., 0.5 M NaCl). However, the efficiency of extraction is often greatly affected by the pH of the buffer and the presence or absence of chelating agents such as EDTA and EGTA. Extraction of membrane-bound and hydrophobic proteins is less affected by the ionic strength of the lysis buffer but often requires a mixture of ionic and nonionic detergents.

When attempting to solubilize a protein for the first time, there are two different strategies that can be employed. At one extreme, harsh conditions can be used in an effort to ensure that the protein is released quantitatively from the cells; however, this may result in loss of immunoreactivity. At the other extreme, gentle conditions can be used to help preserve the protein in a native state; however, this may result in inefficient extraction of the protein from the cells. Which of these approaches should be used is determined in large part by the properties of the antiserum available for immunoprecipitation. For example, as discussed earlier in this chapter, monospecific antisera raised against synthetic peptides may react only with denatured forms of the target protein, whereas monoclonal antibodies directed against native epitopes may be specific for the correctly folded form of the protein. To minimize problems, try to use polyclonal antisera or mixtures of monoclonal antibodies that react with all forms of the protein. It is usually then possible to tailor the extraction conditions to fit the characteristics of the target protein rather than the properties of the available antisera.

Many methods of solubilization, particularly those that involve mechanical disruption of cells, release intracellular proteases that can digest the target protein. The susceptibility of different proteins to attack by proteases varies widely, with cell-surface and secreted proteins generally being more resistant than intracellular proteins. Denatured proteins are much more likely to be degraded than native proteins. It is therefore advisable to take steps to minimize proteolytic activity in cell extracts, especially when harsh conditions of extraction are used. It is important to keep the extracts cold (i.e., at 0°C or below, depending on the sensitivity of the target protein to freezing and thawing). In addition, inhibitors of proteases are commonly included in lysis buffers (Table 18.1). For additional information about inhibitors of proteases, see Barrett and Salvesen (1986).

TABLE 18.1 Properties of Commonly Used Protease Inhibitors

Inhibitor	Active against	Inactive against	Effective concentration	Stock solution
Aprotinin[a] (Trasylol)	kallikrein trypsin chymotrypsin plasmin	papain	1–2 μg/ml	10 mg/ml in 0.01 M HEPES (pH 8.0)
Leupeptins (supplied as a 3:1 mixture of propionyl and acetyl derivatives)	plasmin trypsin papain cathepsin B	chymotrypsin pepsin cathepsins A and D	1–2 μg/ml	10 mg/ml in water
Pepstatin A	pepsin cathepsin D	trypsin plasmin chymotrypsin elastase thermolysin	1 μg/ml	1 mg/ml in ethanol
Antipain	cathepsins A and B papain trypsin	plasmin chymotrypsin pepsin	1–2 μg/ml	1 mg/ml in water
PMSF[b] (phenylmethyl-sulfonyl fluoride)	chymotrypsin trypsin		100 μg/ml	1.74 mg/ml (10 mM) in isopropanol
TLCK (tosyllysine chloromethyl ketone)	trypsin	chymotrypsin	50 μg/ml	1 mg/ml in 0.05 M sodium acetate (pH 5.0)
TPCK (tosylphenylalanine chloromethyl ketone)	chymotrypsin	trypsin	100 μg/ml	3 mg/ml in ethanol
EDTA	metalloproteases		1 mM	0.5 M in water

[a]Aprotinin is a basic polypeptide of 58 amino acids that aggregates if repeatedly frozen and thawed (for review, see Trautschold et al. 1967). The stock solution should be stored in small aliquots at −20°C. Each aliquot should be discarded after use.

[b]**Caution:** PMSF is extremely destructive to the mucous membranes of the respiratory tract, eyes, and skin. PMSF may be fatal if inhaled, swallowed, or absorbed through the skin. In case of contact, immediately flush eyes or skin with copious amounts of water. Discard contaminated clothing.

PMSF is inactivated in aqueous solutions. The rate of inactivation increases with pH and is faster at 25°C than at 4°C. The half-life of a 20 μM aqueous solution of PMSF is about 35 minutes at pH 8.0 (James 1978). This means that solutions of PMSF can be safely discarded after they have been rendered alkaline (pH > 8.6) and stored for several hours at room temperature.

The lysis buffers that are commonly used to prepare extracts of mammalian cells for immunoprecipitation are shown below. In the absence of any information about the target antigen, we recommend trying the triple- and single-detergent lysis buffers before turning to more specialized methods of extraction.

Triple-detergent lysis buffer

50 mM Tris·Cl (pH 8.0)
150 mM NaCl
0.02% sodium azide
0.1% SDS
100 μg/ml phenylmethylsulfonyl fluoride (PMSF)
1 μg/ml aprotinin
1% Nonidet P-40 (NP-40)
0.5% sodium deoxycholate

Single-detergent lysis buffer

50 mM Tris·Cl (pH 8.0)
150 mM NaCl
0.02% sodium azide
100 μg/ml PMSF
1 μg/ml aprotinin
1% Triton X-100 *or* 1% NP-40

High-salt lysis buffer

50 mM HEPES (pH 7.0)
500 mM NaCl
1% NP-40
1 μg/ml aprotinin
100 μg/ml PMSF

No-salt lysis buffer

50 mM HEPES (pH 7.0)
1% NP-40
1 μg/ml aprotinin
100 μg/ml PMSF

Cautions: PMSF is extremely destructive to the mucous membranes of the respiratory tract, the eyes, and skin. PMSF may be fatal if inhaled, swallowed, or absorbed through the skin. In case of contact, immediately flush eyes or skin with copious amounts of water. Discard contaminated clothing.

Sodium azide is poisonous. It should be handled with great care wearing gloves, and solutions containing it should be clearly marked.

PMSF, which is labile in aqueous solution, should be added from a stock solution just before the lysis buffer is used. The rate of inactivation in aqueous solution increases with pH and is faster at 25°C than at 4°C. The half-life of a 20 μM aqueous solution of PMSF is about 35 minutes at pH 8.0

(James 1978). This means that aqueous solutions of PMSF can be safely discarded after they have been rendered alkaline (pH > 8.6) and stored for several hours at room temperature. PMSF is usually stored as a 10 mM or 100 mM stock solution (1.74 or 17.4 mg/ml in isopropanol) at −20°C.

1. Add the lysis buffer of choice (cooled to 0°C) to chilled, washed cell monolayers (step 5, page 18.28). Incubate for 20 minutes on a flat aluminum tray on a bed of crushed ice.

Volume of lysis buffer (ml)	Size of petri dish or well (mm)
1.0	90
0.5	60
0.25	35
0.25	30 (well)

2. Scrape the cells to one side of the dish with a policeman. Using an automatic pipetting device, transfer the cell debris and lysis buffer to a chilled microfuge tube.

   The best policemen are made as follows:
   a. Using a scalpel, slice silicon rubber stoppers (2 cm in diameter) into quarters.
   b. Place each piece of stopper on the bench and impale it on a pasteur pipette.
   c. Use the straight edge of the sliced stopper for scraping cells.
   d. After use, store the policemen in detergent solution before washing. The policemen can be sterilized by autoclaving for 20 minutes at 15 lb/sq. in.

3. Centrifuge the lysate at 12,000*g* for 2 minutes at 4°C in a microfuge.

4. Transfer the supernatant to a fresh microfuge tube, and store it on ice or at −70°C, depending on the sensitivity of the target antigen to freezing and thawing.

Notes

i. When experimenting with conditions for solubilization of the protein of interest, it is sometimes useful to extract the cell debris (step 4) with a stronger lysis buffer.

ii. Cells growing in suspension should be concentrated by centrifugation and then resuspended in the appropriate lysis buffer (chilled to 0°C) at a concentration of 10^7 cells/ml. After 30 minutes at 0°C, transfer the cell suspension to a chilled microfuge tube and proceed as described in step 3.

iii. After thawing, samples that have been stored at −20°C should be centrifuged at 12,000*g* for 5 minutes at 0°C in a microfuge. This removes aggregates of cytoskeletal elements.

MECHANICAL LYSIS OF YEAST

This method can be used only when the antibody recognizes denatured forms of the target protein.

1. Collect the cells from a 1-ml culture by centrifugation at 12,000*g* for 1 minute at 0°C in a microfuge or at 3000 rpm for 5 minutes at 0°C in a Sorvall RT6000 (or equivalent). Discard the supernatant (unless the protein of interest is a secretory protein).

2. Resuspend the cells by vortexing in 1 ml of ice-cold 50 mM Tris · Cl (pH 8.0). Recover the cells by centrifugation as described in step 1. Pour off the supernatant, and remove as much of the residual fluid as possible with a disposable pipette tip attached to a vacuum line.

3. Resuspend the pellet in 5 volumes (~250 μl) of yeast lysis buffer.

Yeast lysis buffer
50 mM Tris · Cl (pH 8.0)
0.1% Triton X-100
0.5% SDS

4. Add acid-washed glass beads (0.45–0.50 mm in diameter) to the level of the meniscus. The total volume of the mixture of glass beads and yeast lysis buffer should be approximately 400 μl.

   At one time, glass beads of the correct size could be purchased only from one supplier, located in Germany (B. Braun, Melsüngen). Recently, however, several companies in the United States have begun to sell beads of the correct size of acceptable quality.

5. Vortex the suspension on a heavy-duty vortexing machine for five periods of 20 seconds each. Cool the suspension on ice for 1 minute between each cycle of vortexing. Monitor cell lysis by phase-contrast microscopy.

6. Recover the cell extract by punching a small hole through the bottom of the microfuge tube. This is best done with a red-hot hypodermic needle (23 gauge). Place the microfuge tube containing the glass beads inside an empty microfuge tube. Centrifuge the piggybacked pair of tubes in a 15-ml Corex tube at 2000*g* for 2 minutes at 4°C.

7. Recover the bottom microfuge tube, which should contain the cell extract. Clarify the extract by centrifuging the tube at 12,000*g* for 5 minutes at 4°C in a microfuge. Transfer the supernatant to a fresh microfuge tube.

   Extracts of yeast cells are usually stable when stored at −20°C. However, if the protein of interest is sensitive to proteolysis, add protease inhibitors to the extracts (see Table 18.1) and store them at 0°C. Try to minimize the time between cell lysis and immunoprecipitation.

8. Immunoprecipitate the protein of interest from the cell extract as described on pages 18.44–18.46.

This method is used when the available antibody recognizes the native form of the target protein.

1. Collect the cells from a 1-ml culture by centrifugation at 12,000*g* for 1 minute at 0°C in a microfuge or at 3000 rpm for 5 minutes at 0°C in a Sorvall RT6000 (or equivalent). Discard the supernatant (unless the protein of interest is a secretory protein).

2. Resuspend the cells by vortexing in ice-cold phosphate-buffered saline (PBS; see Appendix B). Recover the cells by centrifugation as described in step 1. Discard the supernatant.

3. Resuspend the pellet in a volume of stabilizing buffer A (at room temperature) equivalent to the volume of the original culture (usually 1 ml as in step 1). Incubate the supension for 10 minutes at 30°C.

Stabilizing buffer A
1 M sorbitol
10 mM $MgCl_2$
2 mM dithiothreitol
50 mM potassium phosphate (pH 7.8)
100 μg/ml phenylmethylsulfonyl fluoride (PMSF)

Caution: PMSF is extremely destructive to the mucous membranes of the respiratory tract, the eyes, and skin. PMSF may be fatal if inhaled, swallowed, or absorbed through the skin. In case of contact, immediately flush eyes or skin with copious amounts of water. Discard contaminated clothing.

PMSF, which is labile in aqueous solution, should be added from a stock solution just before the stabilizing buffer is used. The rate of inactivation in aqueous solution increases with pH and is faster at 25°C than at 4°C. The half-life of a 20 μM aqueous solution of PMSF is about 35 minutes at pH 8.0 (James 1978). This means that aqueous solutions of PMSF can be safely discarded after they have been rendered alkaline (pH >8.6) and stored for several hours at room temperature. PMSF is usually stored as a 10 mM or 100 mM stock solution (1.74 or 17.4 mg/ml in isopropanol) at −20°C.

4. Recover the cells by centrifugation as described in step 1, and resuspend the cell pellet in a volume of stabilizing buffer B equal to the volume of the original culture. Incubate the suspension for 2 minutes in a water bath set at 30°C.

Stabilizing buffer B

1 M sorbitol
10 mM $MgCl_2$
2 mM dithiothreitol
25 mM potassium phosphate (pH 7.8)
25 mM sodium succinate (pH 5.5)
100 µg/ml PMSF

5. Add 0.25 volume of a stock solution of zymolase 100T (10 mg/ml).

   Zymolase is an enzyme, isolated from cultures of *Arthrobacter luteus*, that catalyzes lysis of cell walls by hydrolyzing glucose polymers with 1→3 linkages. As supplied by commercial manufacturers, zymolase 100T is contaminated with other enzymes, including proteases. It should therefore be used in conjunction with protease inhibitors.

6. Incubate the suspension at 30°C. After 15 minutes, take out two small aliquots of the suspension. To one sample, add Nonidet P-40 (NP-40) to a final concentration of 1%. Examine the two samples by phase-contrast microscopy. Protoplasts formed by the action of zymolase will be visible in the untreated sample but should be lysed in the sample containing detergent. Continue incubation until the majority of the cells have been converted to protoplasts. This usually takes 20–30 minutes.

7. Collect the protoplasts by centrifugation at 500*g* for 10 minutes at 4°C. Discard the supernatant.

8. Resuspend the pellet (which consists of protoplasts and intact cells) in a volume of lysis buffer equivalent to 0.01 volume of the original culture. Store the suspension at 0°C for 30 minutes.

   Either NP-40 high- or no-salt lysis buffer (see page 18.32) or RIPA buffer (see page 18.38) may be used, depending on the properties of the available antibody. It is essential to include protease inhibitors in the lysis buffer (see Table 18.1).

9. Centrifuge the suspension at 12,000*g* for 10 minutes at 4°C in a microfuge. Transfer the supernatant to a fresh microfuge tube.

   Extracts of yeast cells are usually stable when stored at −20°C. However, if the protein of interest is sensitive to proteolysis, add protease inhibitors to the extracts (see Table 18.1) and store them at 0°C. Try to minimize the time between cell lysis and immunoprecipitation.

10. Immunoprecipitate the protein of interest from the cell extract as described on pages 18.44–18.46.

Note

When experimenting with conditions for solubilization of the protein of interest, it is sometimes useful to extract the cell debris (step 9) with a stronger lysis buffer.

RAPID LYSIS OF YEAST CELLS

This method can be used only when the antibody recognizes denatured forms of the target protein.

1. Collect the cells from a 1-ml culture by centrifugation at 12,000*g* for 1 minute at 0°C in a microfuge or at 3000 rpm for 5 minutes at 0°C in a Sorvall RT6000 (or equivalent). Discard the supernatant (unless the protein of interest is a secretory protein).

2. Resuspend the cells in 0.5 ml of 25% trichloroacetic acid (TCA).

3. Recover the cells by centrifugation as described in step 1, and resuspend them in 1 ml of 90% acetone.

4. Recover the cells by centrifugation as described in step 1, and estimate the volume of the pellet. Resuspend the pellet in an equal volume of 1% SDS.

5. Add acid-washed glass beads (0.45–0.50 mm in diameter) to the level of the meniscus.

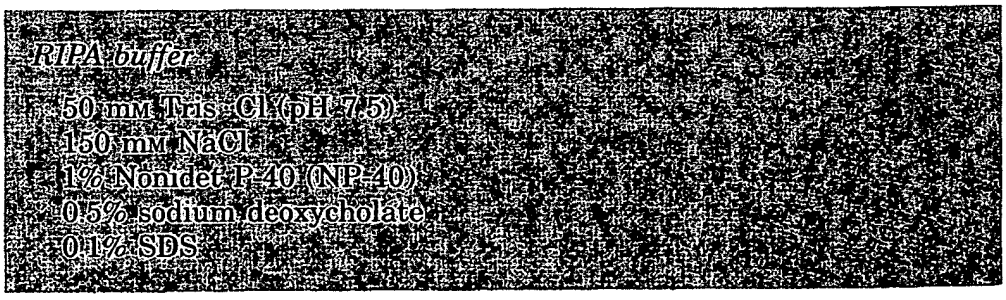
   At one time, glass beads of the correct size could be purchased only from one supplier, located in Germany (B. Braun, Melsüngen). Recently, however, several companies in the United States have begun to sell beads of the correct size of acceptable quality.

6. Vortex the suspension on a heavy-duty vortexing machine for five periods of 20 seconds each. Cool the suspension on ice for 1 minute between each cycle of vortexing. Monitor cell lysis by phase-contrast microscopy.

7. Using a red-hot hypodermic needle (23 gauge), make a hole in the lid of the microfuge tube. This prevents the glass beads from exploding out of the tube during heating.

8. Heat the tube in a boiling-water bath for 3 minutes.

9. Transfer the glass beads into a fresh microfuge tube, and wash the original tube with 0.5 ml of RIPA buffer. Add the wash to the fresh microfuge tube containing the glass beads.



10. Recover the cell extract by punching a small hole through the bottom of

the microfuge tube. This is best done with a red-hot hypodermic needle (23 gauge). Place the microfuge tube containing the glass beads inside an empty microfuge tube. Centrifuge the piggybacked pair of tubes in a 15-ml Corex tube at 2000*g* for 2 minutes at 4°C.

11. Recover the bottom microfuge tube, which should contain the cell extract. Clarify the extract by centrifuging the tube at 12,000*g* for 5 minutes at 4°C in a microfuge. Transfer the supernatant to a fresh microfuge tube.

    Extracts of yeast cells are usually stable when stored at −20°C. However, if the protein of interest is sensitive to proteolysis, add protease inhibitors to the extracts (see Table 18.1) and store them at 0°C. Try to minimize the time between cell lysis and immunoprecipitation.

12. Immunoprecipitate the protein of interest from the cell extract as described on pages 18.44–18.46.

LYSIS OF BACTERIA

Immunoprecipitation of target proteins from cell lysates is used far more rarely with bacterial expression systems than with mammalian expression systems because foreign proteins are usually expressed in *E. coli* from powerful, inducible promoters. Following induction, therefore, novel gene products frequently accumulate to such a level that they can be detected by the appearance of novel bands after SDS-polyacrylamide gel electrophoresis of total cell proteins. Immunological identification of these novel bands can then be obtained by western blotting using antisera or monoclonal antibodies that react with denatured forms of the target protein. If no novel proteins are detectable by this technique, it makes more sense to test the cloned DNA in a series of different *E. coli* expression systems rather than to attempt immunoprecipitation from cell extracts that contain unusably small quantities of target protein.

Total lysates of small-scale cultures of *E. coli* are easily made as follows:

1. After induction for the appropriate period of time, recover the bacteria from 1 ml of culture by centrifugation at 12,000*g* for 30 seconds in a microfuge.

2. Remove the medium by aspiration, and then resuspend the pellet by vortexing in 0.5 ml of ice-cold 50 mM Tris · Cl (pH 7.4). Recover the bacteria by centrifugation at 12,000*g* for 30 seconds at 0°C in a microfuge.

3. Remove the supernatant by aspiration, leaving the bacterial pellet as dry as possible. Take care to remove any beads of fluid that adhere to the sides of the microfuge tube.

4. Resuspend the pellet by vortexing in 25 μl of H_2O. As soon as the pellet is dispersed, add 25 μl of 2 × SDS gel-loading buffer and continue vortexing for 20 seconds.

2 × SDS gel-loading buffer

100 mM Tris · Cl (pH 6.8)
200 mM dithiothreitol
4% SDS (electrophoresis grade)
0.2% bromophenol blue
20% glycerol

2 × SDS gel-loading buffer lacking dithiothreitol can be stored at room temperature. Dithiothreitol should then be added just before the buffer is used, from a 1 M stock (see Appendix B).

5. Place the sample in a boiling-water bath for 5 minutes.

6. Shear the chromosomal DNA by sonication, using either a sonicator with an immersible tip or a sonicator that can process many samples simulta-

neously in a chilled cup. Depending on the power output of the sonicator and its state of tuning, between 30 seconds and 2 minutes at full power should be sufficient to reduce the viscous lysate to manageable levels.

Larger samples can be sheared by repeated passage through a 23-gauge hypodermic needle.

7. Centrifuge the sample at 12,000*g* for 10 minutes at room temperature. Transfer the supernatant to a fresh tube and discard the pellet.

8. Analyze 25 μl of the sheared or sonicated lysate by electrophoresis through an SDS-polyacrylamide gel as described on pages 18.47–18.54. Store the unused portion of the sample at −20°C.